The Influence of Fungi Upon Soil Structure and Soil Water Relations

A thesis submitted in partial fulfilment of the requirements of the University of Abertay Dundee for the degree of Doctor of Philosophy

by

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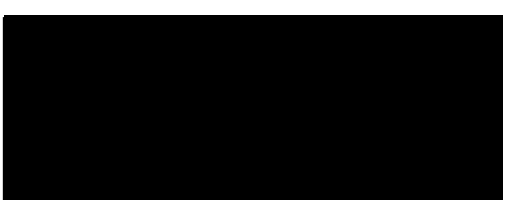
I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed ... [redacted] ..... Date 4th June 2004

Declaration

I hereby declare that this thesis has been composed by myself and that it has not been accepted in any previous application for a degree. The work of which it is a record, is my own, unless otherwise stated. All verbatims have been distinguished by quotation marks and sources of information specifically acknowledged by means of references.



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Abstract

The investigation of soil structural stability and soil water processes was assessed through the application of laboratory investigations and a field based analysis. The impact of an arbuscular mycorrhizal (AM) fungal exudate glomalin (a glycoprotein), proposed to be hydrophobic was assessed for a correlation with low levels of soil hydrophobicity through measures of subcritical water repellency. Initially no correlation was reported but a further temporal investigation that involved a soil inoculum detected a significant positive effect; the results indicated that a certain concentration of protein is required before an influence upon soil hydrophobicity is detected.

The temporal investigation detected significant re-aggregation of previously disturbed soil; this was linked to both increases in fungal biomass and enmeshment by plant roots. Soil in the direct vicinity of plant roots showed the most significant increases in aggregated structures, indicating that plant root enmeshment was one of the predominant factors in soil aggregation. Soil water repellency was directly correlated with measures of macroaggregates (aggregates >2000 μm), indicating that increased hydrophobicity is a mechanism involved in aggregate stabilisation.

Field scale sampling and analysis indicated that fertilizer applications had varied effects upon fungal populations, dependent on the particular land management applied to the soil. Undisturbed grassland where fungal biomass was likely to be the predominant microorganism present showed significant effects of fertilizer regime upon fungal biomass, with effects likely to be related to plant-fungi interactions through changes in AM fungal biomass. The influence of fertilizer regime on arable sites was less pronounced which indicated a significant influence of disturbance reducing fungal biomass and reducing the direct and indirect effects associated with fertilizer additions.

The investigation of soil pore spatial distribution is essential for understanding soil processes as water flow, gas and nutrient exchanges will occur within pore space, as will many biological processes. The investigation of inter-aggregate pore space was completed upon soil aggregates ≤2 mm that had been exposed to previous experimental perturbations, where increased aggregate stability, water repellency and fungal biomass were reported. A resolution of ≈4 μm was achieved and changes in percentage porosity and spatial pore distributions were detected as a result of direct and indirect effects of plant roots. Greatest increases in heterogeneity of pore space were reported in soil from close proximity to roots, with a reduction in this phenomenon at an increasing distance from the root zone. The mechanism proposed for these changes was localised drying from roots.

The results presented provide greater understanding of controlling factors associated with soil water and stability mechanisms, along with demonstrating biologically and physically induced changes in micro and meso-scale structures as a result of different treatments. The work provides scope for further investigation of particular biological and physical factors associated with soil structural mechanisms.

Chapter 1: Literature Review

1.1 Introduction

Soil structure can be defined as the aggregation or arrangement of primary particles in the soil (Brady, 1974). The stability and resultant pore structure of aggregated soil particles will ultimately influence soil processes (Young and Crawford, 1991). There are many factors associated with maintenance of a structurally healthy soil, with extensive research investigating the role that microorganisms play in the stabilisation of soil particles (Jastrow *et al.*, 1998; Tisdall, 1994; Tisdall *et al.*, 1978). The microbial community within a soil and the complex interactions that result from manipulations to the soil, such as aboveground vegetation (Coleman, 1985), disturbance and nutritional inputs (Young and Ritz, 2000), are still not yet fully understood. Additionally changes to the microbial community may have direct and indirect consequences upon soil structural stability.

Fungi are well documented in their ability to stabilise soil particles (Jastrow *et al.*, 1998; Tisdall and Oades, 1982), and the relationship between fungal hyphae and the physical assessment of soil through investigations of soil aggregates has been studied extensively (Bossuyt *et al.*, 2001; Denef *et al.*, 2001; Jastrow *et al.*, 1998; Monlope *et al.*, 1987). Much of the research involves the assessment of different sized aggregates and their coherence in water, factors which are unlikely to occur in the field. Such rather artificial means of analysing soil structures may lead to incorrect assumptions about factors implicated in the stabilisation of soil *in situ*.

Fungi are also reported to change the wetting properties of soils (Bond and Harris, 1964; Doerr *et al.*, 2000; Hallett *et al.*, 2002; Savage *et al.*, 1969). Impeded or more controlled wetting of soil is a process associated directly with structurally stable soils. The direct assessment of this mechanism is a factor that is frequently neglected from the analysis of soil stability and perhaps an important flaw in the investigation of soil biophysics.

This thesis will investigate mechanisms associated with soil structural stability, such as water infiltration, and comparing such measures with more traditional measurements allowing a balanced view of how the measurements are related and

factors that are associated. This Chapter aims to review research surrounding factors involved in the creation and maintenance of soil structure. The main focus will be on the impact of soil fungi, and this will be discussed in relation to water flow, and structural properties from laboratory and field based work. Discussion will initially centre on defining what stable soil structure is, and how this can be assessed, along with detailing how microorganisms (in particular fungi) in soil can be quantified, or observed, to study their role in soil stability and enhancement.

1.2 Biological influences upon soil

The primary components of soil, minerals, silt, sand and clay, combine with organic matter to create the most biologically diverse ecosystem on earth (Brady, 1974). Much of this ecosystem complexity is driven by multifaceted root-soil-microbial-faunal interactions (Brown, 1975; Coleman, 1985; Plaster, 1997). Interactions within the soil, particularly microbial, are responsible for a number of essential soil processes, for example nutrient cycling. Soil plays a key role in the completion of cycling major elements including carbon, nitrogen, phosphorus and sulphur, which are essential for biological systems (Bosatta and Berendse, 1984; Himes, 1998; Nannipieri *et al.*, 1994). Microorganisms such as protozoa, algae, fungi, bacteria and actinomycetes range in scale from 0.5 μm for bacteria, to 30-50 μm for protozoa, whilst fungal hyphae may form hyphal networks meters in length (Giller, 1996; Hattori, 1976). The presence of microbial populations allows essential recycling of organic material through microbial decomposition.

Larger soil animals such as arthropods, nematodes, enchytraeid worms and earthworms are important to the soil ecosystem and their effects are well documented (Brady, 1974; Russell, 1988). The most numerous soil organisms are bacteria and actinomycetes, but due to their size they constitute only one third of the soil biomass (Lyda, 1981). Bacteria tend to inhabit small pores within soil, which retain water even in dry conditions (Foster, 1988), or often occur within aggregates, whilst fungi are generally restricted to larger pores between aggregates (Kilbertus, 1980).

Microbial populations are responsible, along with plants, for creating and enhancing stable soil physical structure (Degens, 1997; Giller, 1996; Jastrow and Miller, 1991; Jastrow *et al.*, 1998; Miller and Jastrow, 2000; Oades, 1993; Tisdall and Oades, 1982; Tisdall *et al.*, 1997).

1.2.1 Defining soil structure

The primary mineral particles of soil (i.e. sand, silt and clay) are bound together by a combination of electrostatic forces, capillary forces from water, and biological material.

Aggregation of soil into stable structures is essential for soil to withstand physical stresses with minimal disruption. Structural stability is defined as the ability of a soil to maintain its arrangement of solids and pores when exposed to stress (Oades, 1993). Stresses upon soil can come from a number of sources including compaction and slaking. Here slaking is discussed, as it occurs under natural weathering processes and is probably the stabilising mechanism in soil most affected by biological processes. Slaking occurs when water inundation causes a rapid build-up of air pressure in initially dry soil. As the air from within the pore space tries to escape, the increased pressure may be enough to destroy intact structural units such as soil aggregates (Hillel, 1998). The result of slaking is that aggregates are often broken down into smaller particles increasing the risk of soil crusting (Le Bissonnais *et al.*, 1998; Levy *et al.*, 1997), which in turn can increase soil erosion (Le Bissonnais *et al.*, 2002; Le Bissonnais and Singer, 1993; Levy *et al.*, 1997). The stability of soil against slaking is also essential for the protection of soil organic matter. Protected organic matter released from slaked soil aggregates may be subjected to rapid decomposition (Christensen, 1996). In addition to slaking, other processes of physical disruption to soil aggregates include the differential swelling of clays, disruption of physiochemical associations between particles and the mechanical dispersion of soil due to the physical impact of water (Le Bissonnais, 1996).

1.2.2 Organisation and stabilisation of soil structure

Concepts relating to the organisation and aggregation of soil by biological and physical forces were reviewed by Tisdall and Oades (1982). They produced one of the most cited references in the field of soil science by proposing a conceptual model of an aggregate hierarchy. The aggregate hierarchy model suggests that the soil is not homogeneous, and is made up of organised aggregated particles, at a range of scales, held together by organic and inorganic materials (Tisdall, 1996).

Although the theoretical approach proposed by Tisdall and Oades (1982) suggests a breakdown of soil size classes, there are conflicting views of the validity of studying soil in this way. Letey (1991) suggested that laboratory based experiments "*frequently on fragmented samples, do not always lead to an understanding of the functionality of soil structure under field conditions*". Young *et al.* (2001) suggested that the soil profile is not made up separate aggregate structures but "*typically the undisturbed profile in reality exists as a continuous convoluted pore*". Tisdall and Oades (1982) studied soil as an aggregated hierarchical structure as opposed to a number of separate hierarchical structures within the soil, an important distinction. Binding agents within soil at larger scales are mainly organic and inorganic at smaller scales. Tisdall and Oades (1982) described binding agents on the basis of their size and age as either transient, temporary or persistent. Whether related to an aggregate framework, or alternative views on the organisation of soil structure, the view of Tisdall and Oades (1982) on binding agents is still valid.

Transient binding agents

These agents are short-lived and are readily decomposable. Roots, fungi and bacteria produce extra-cellular mucilages, which are mainly polysaccharide but can also contain polyuronic acids and amino acids (Tisdall, 1996), and act as glues within the soil. Plants tend to produce polysaccharides, which are then broken down by the microbial population and thus replaced by microbial polysaccharides (Tisdall, 1996). Microbial polysaccharides can bind soil particles together through polymer bridges (Greenland, 1972). Bacterial and fungal polysaccharides including xanthan, dextran and scleroglucan, which, upon absorption onto clay minerals, can induce,

increased water stability. Additionally these exudates may buffer against water fluctuations (Chenu, 1993; Chenu *et al.*, 1987). The binding influence of these substances along with the associated scales is illustrated in Fig. 1.1.

In addition to biological binding agents, aluminium oxides are also reported to aggregate soil in a very stable form, with breakdown not occurring naturally and only in the laboratory when exposed to ultrasound (Oades and Waters 1991; Tippkötter, 1993).

Temporary binding agents

Temporary binding agents include plant roots and fungal hyphae. These agents are dependent on the land management practice used. Usually lasting for weeks or months (Tisdall and Oades, 1982), these binding agents move through the soil as plants grow and develop. Roots act as binding agents both by enmeshing soil particles and by providing a source of organic residues that create and support large associated microbial populations (Jastrow *et al.*, 1998; Miller and Jastrow, 1990; Tisdall and Oades, 1982). Temporary binding agents can also include cements that hold particles together in a solid state, even when wet, and do not rely on entangling particles together. These cements include plant and fungal debris, crystalline oxides and highly disordered aluminosilicates (Tisdall and Oades, 1982).

Persistent binding agents

Persistent soil binding agents are mainly humic substances and some metals, but also include electrostatic forces between particles. They form chemical associations with soil particles, which create incredibly strong physical bonds that can only be disrupted by sonic disturbance in the laboratory (Tisdall and Oades, 1982). Persistent binding agents are responsible for the formation of structures less than 2 μm. At this scale, aggregates consist of individual clay plates gathered together in floccules. The flocculation of clay plates arises as a result of a number of forces causing an attraction between plates. Van der Waal's forces, H-bonding and coulombic attraction are all electrostatic forces drawing particles together (Tisdall

and Oades, 1982), and allow the formation of organo-mineral interactions between clay plates.

In addition to the physical binding of soil proposed by Tisdall and Oades (1982), soil biota can also maintain soil structure through water-mediated changes to the soil. A summary of processes involved in soil structure enhancements is shown in Fig. 1.1

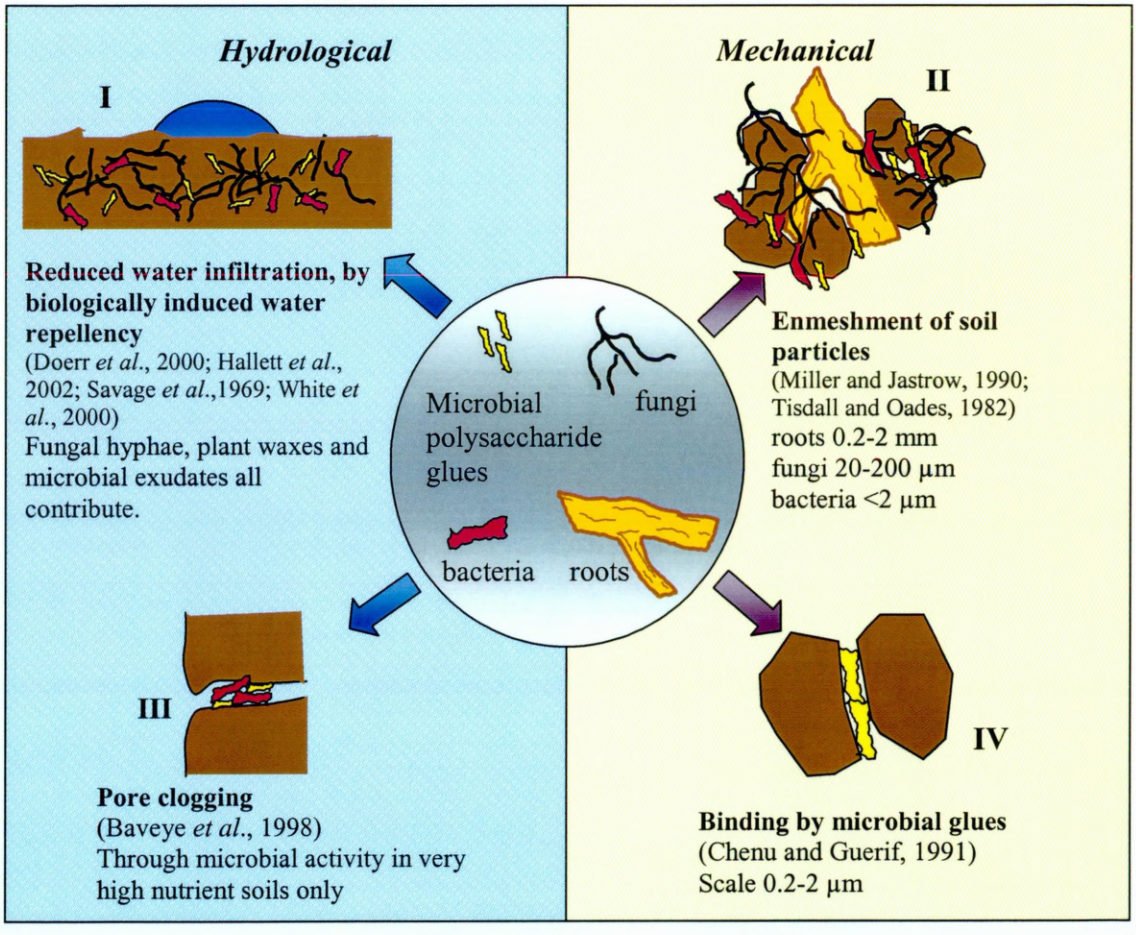


Fig. 1.1 Biological influences upon soil structure, illustrating both mechanical and hydrological mechanisms associated with soil structure (not to scale).

The mechanical stabilisation of soil by biota (II & IV in Fig 1.1) is a key factor in most soils and is well documented (Jastrow *et al.*, 1998; Miller and Jastrow, 1990 & 2000; Monlope *et al.*, 1987; Tisdall *et al.*, 1978 & 1997; Tisdall and Oades, 1979 &

1982). The level of infiltration of water into soil is a mechanism directly related to the maintenance of soil stability. Reductions in water infiltration in dry soil by water repellency is probably very common (Hallett and Young, 1999); however, this approach is less reported than a mechanical assessment of soil structure. Pore clogging is likely to occur only in systems that have a high substrate loading such as sewage sludge (Baveye *et al.*, 1998).

1.2.3 The influence of fungi upon structural stabilisation of soils

Fungal populations in soil are particularly important binding agents (Andrade *et al.*, 1998; Bearden and Petersen, 2000; Miller and Jastrow, 2000; Thomas *et al.*, 1993). Whilst a single fungal hypha is a relatively weak structure a dense fungal mycelium, and fungi associated with roots, may form extensive networks creating a stable web enmeshing soil particles (Tisdall and Oades, 1982). Jastrow *et al.* (1998) used a field-based investigation to study a range of factors involved in soil stability. They made measurements of arbuscular mycorrhizal (AM) fungal length, fine and very fine root lengths, and carbon chemistry to assess how they affect aggregate stability (of structures <250 μm). Using Path Analysis, Jastrow *et al.* (1998) assessed the extent to which various field measures contributed to the physical stabilisation of soil (Fig. 1.2). This work drew on important distinctions between 1st and 2nd order effects of variables on structural stability. The key to an adequate understanding of soil stabilisation is establishing how the direct and indirect pathways interact under varying environmental conditions.

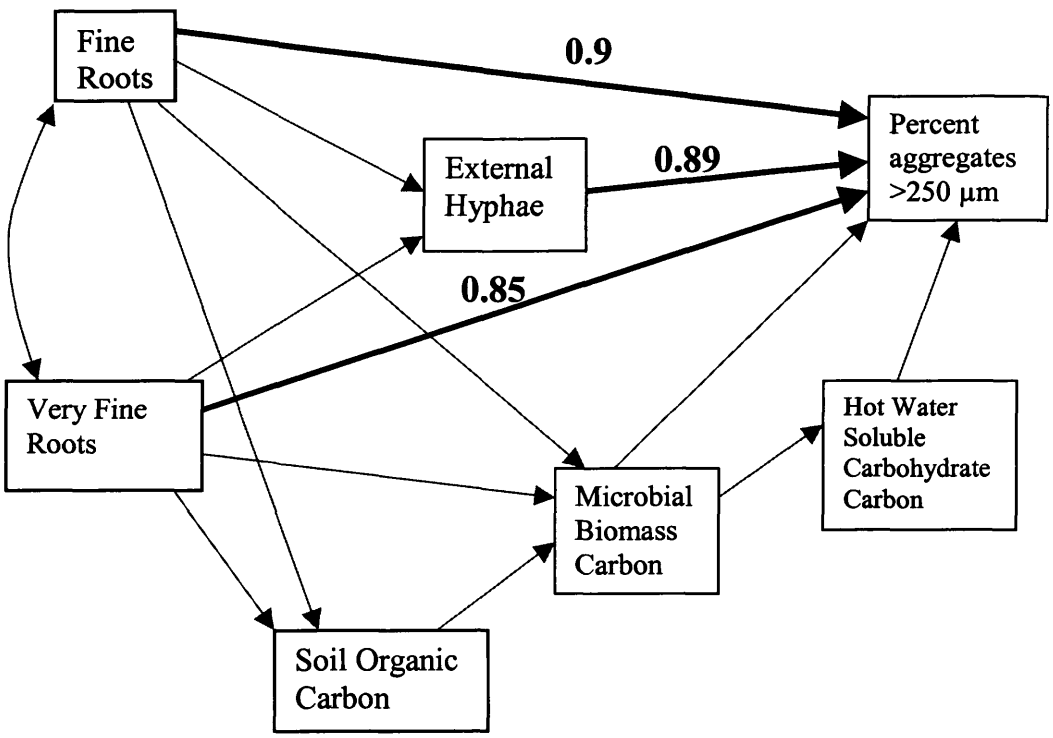


Fig. 1.2 A conceptual path of relationships between root lengths, mycorrhizal fungal hyphae, three different carbon pools, and resulting percentage aggregates [model by Jastrow *et al.* (1998)]. Bold lines indicate those relationships with the strongest direct influence upon aggregate formation; the values are Pearson product-moment correlation "*r*" values.

The ability of fungi to enhance soil structure is significant and well documented (Jastrow and Miller, 1991; Miller and Jastrow, 1990 & 1992; Monlope *et al.*, 1987; Tisdall, 1991; Tisdall *et al.*, 1997). Miller and Jastrow (1992) proposed that AM fungi aggregate soil by initially entangling primary particles, the combined effects of roots and hyphae aggregate the particles into structures increasing both in stability and complexity. This will not however be the only mechanism applied to soil stabilisation, as illustrated in Fig. 1.1. Changes to soil water processes by fungal hyphae are often overlooked in investigations of soil aggregate stabilisation, with many researchers utilising a solitary means of assessing soil stability. Fungal hyphae exude polysaccharides (Miller and Jastrow, 1992) and other proteinaceous substances (Wright and Upadhyaya, 1998; and Section 1.4.2), which ultimately may also contribute to soil stability.

Bond and Harris (1964) reported that fungal hyphae may change the wetting behaviour of soils, and Guggenberger *et al.* (1999) proposed that in addition to physical entanglement, fungi were responsible for impeded wetting of soils, in some cases slowing water infiltration, offering a more controlled uptake of water and potentially structurally enhancing the soil. Both Savage *et al.* (1972) using *Penicillium* spp, and White *et al.* (2000) using *P.chrysosporium* and *C.versicolor*, reported changes in water infiltration induced by the presence of fungi. Clothier *et al.* (2000) also proposed that soil water repellency in an experimental soil was induced (among other factors) due to the presence of AM fungi (a process that will be explained in more detail in Section 1.3.4).

A detailed literature review of how land management impacts upon fungal populations is given in Section 1.5. The nature of interactions in the soil environment is a combination of biological and physical properties; a review of some of the methodologies displayed in Fig. 1.1 and the associated biological mechanisms, in particular fungal impacts, within soils will be discussed as a means to the investigation of biological and physical interactions in soil.

1.3 Methods of assessing soil structure

Soil physical stability tests commonly assess stability against wetting stresses by immersion in water. They attempt to mimic field situations that arise under rainfall when the physical structure is susceptible to disruption. Proposed theoretical differences in scale can be examined by quantifying different sized aggregates produced by these tests, in order to try to physically measure differences in structure. The direct mechanical assessment of soil through a friability index from tensile strength can be assessed as an indicator of structural quality (Utomo and Dexter, 1981), however this method will not be reviewed in the section that follows.

1.3.1 Particle size distribution

The varying proportions of different sized mineral particles can be measured through particle size distribution analysis. At the smallest scale are clays, generally grouped together as a result of chemical associations; next are sand and silt which do not readily form these chemical associations and are reliant on biological associations. The aggregation of these particles means that the assessment of particle size does not give an indication of biological induced changes in soil structure, as the size and proportion of particles in a soil is not subject to rapid change (Brady, 1974). This is a limitation of this methodology, which may only be used to characterise different soils on the basis of particle size.

Particle size distributions may be assessed using the international pipette method or hydrometer method, which use Stokes' Law to evaluate the sedimentation rate of different sized particles. The international pipette method relies on an artificial means of disaggregation through harsh chemical treatments, which bear no relation to the field situation. More recently, X-ray sedimentation has been used to estimate particle size distribution in soil (Watts *et al.*, 2001). Using Stokes' Law the X-ray attenuation of a soil suspension can be used to calculate the size distribution of aggregates or particles. This method is at the "micro" scale were the only disruption applied to soils is sieving to less then 100 μm prior to analysis, with structure below this scale undisturbed.

1.3.2 Measurement of slaking

As previously reported in (Section 1.2.1) slaking is seen to be an important mechanism. Rapid wetting of soil can cause structural damage through entrapped air (Yoder 1936). Yoder (1936) was one of the first to use the coherence of soil aggregates in water as a means to assess soil structural stability. He described slaking as the initial fragmentation of soil aggregates several millimetres in diameter which may disintegrate further to become microaggregates [<250 μm diameter] (Tisdall, 1991; Oades and Waters, 1991), and used wet sieving as a means to separate aggregates into size fractions. Emerson (1967) further characterised soil

aggregates. Based on the coherence of the aggregates in water, soils were classified into one of eight classes ranging from 1: Slaking & complete dispersion to 8: No slaking or swelling. The aggregate classification method is a visual observation assessed qualitatively by the operator and thus has the potential to introduce operator-to-operator differences. Further advances on this test are measurements of aggregate dispersion through exposure to ultrasonic dispersion. Imeson and Vis (1984) and Tippkötter (1993), in a similar fashion to Emerson (1967), assessed the breakdown of soil aggregates using ultrasonic dispersion. Imeson and Vis (1984) suggested this method was better for structurally stable soils where the breakdown in water may not normally be significant. Both Imeson and Vis (1984) and Tippkötter (1993) reported this method to be highly reproducible as the level of dispersive energy was readily controlled and easily determined.

The examination of soil structure in this way results in a relatively rapid and simple assessment of the stability of soil in water and allows for the observation of changes in soils through management practices. The measurement devised by Emerson (1967) assesses soil structure through the investigation of 3-5 mm aggregates, which is a relatively small scale in relation to the bulk soil. The measurement is a qualitative assessment of soil, and does not allow for a quantitative measurement of particular soil physical processes.

1.3.3 Measures of aggregate stability / size distribution

Most "traditional" research in soil physical measurements assesses stability through the separation of soil into fractions based on the work of Yoder (1936) mentioned previously in Section 1.3.2. On the basis of this work researchers further developed the methodology and assessed aggregate size distribution, aggregate stability or mean weight diameter (MWD) (Van Bavel, 1949). This technique has been used in 641 papers published on soil structure research since 1995 (Source: ISI Web of Science), with Young *et al.* (2001) showing that between 1990-2000 over 60% of all papers on soil structure were related to such measures. The method involves sieving soil to give different fractions (Cambardella and Elliott, 1992; Kemper *et al.*, 1985).

Ranges of size-classes are used with scales dependent on the methodology selected. Typically the soil (either initially wet or air dry) is placed on to a set of nested sieves and disrupted in water for approximately two minutes, until the soil is separated on to each of the sieves. Previous work has shown the importance of fungi in enhancing soil structure, with a plethora of studies relating aggregate stability to soil biological processes, some of which will be reviewed here.

Assessing the effect of the application of a fungicide upon soil structure Denef *et al.* (2001) detected a substantial decline in stabilisation upon exposure to the fungicide reporting that fungi contributed to 66% of the microbial biomass. Bossuyt *et al.* (2001) also reported fungi as the key factor in the formation of macroaggregates (those >250 μm). Denef *et al.* (2001) and Bossuyt *et al.* (2001) demonstrated some of the factors involved in aggregate formation. However, both used a significant physical disruption procedure, with test soil being sieved to less then 250 μm, discarding material >1000 μm, and remixing in material 250-1000 μm, to ensure that no field-based intact aggregates remained in the soil. The removal of large quantities of soil material including organic matter creates a rather artificial set-up. Measures of aggregate size distribution report results at only one scale, >250 μm, indicative that the investigation may have been too specific, providing no other soil physical measurements over a range of scales.

Monlope *et al.* (1987) assessed fungal biomass and bacterial populations over a thirty-day incubatory period and examined their impact upon aggregate stability. After 15 days they found a substantial increase in fungal biomass, which was correlated with temporary increases in aggregate stability. Monlope *et al.* (1987) reported that increased aggregate stability was as a result of hyphal entanglement. After 15 days incubation there was a reduction in fungal biomass, which in turn allowed for a significant increase in bacterial biomass. No correlation between aggregate stability and bacterial biomass was detected. With this finding Monlope *et al.* (1987) hypothesised that polysaccharides exuded by bacteria did not directly affect aggregate stability, with newly exuded polysaccharides simply supplying a fresh substrate rather than physically binding soil particles. The negligible relationship between bacterial biomass and aggregate stability would have been associated with the lack of impact bacteria have upon the wetting properties of soil

(Hallett *et al.*, 2001). Fungal biomass is reported to directly impart soil hydrophobicity (Bond and Harris, 1964; Miller and Wilkinson, 1977; Savage *et al.*, 1969; White *et al.*, 2000; York and Canaway, 2000), and therefore is most likely to be directly associated with resulting aggregate stabilisation. Without a direct measure of this (which is explained comprehensively in Section 1.3.4) the findings of Monlope *et al.* (1987) require further research to establish the mechanisms of fungal hyphae in stabilisation of soil structure. By suppressing bacteria or fungi with biocides, Hallett *et al.* (2001) showed that fungi and not bacteria induced water repellency.

Measures of aggregate stability involve the physical separation of the soil, a factor that is highly unlikely to occur in the field and may be suitable only for disturbed soil environments (Young *et al.*, 2001). Measures of aggregate stability may be used as a surrogate of physical stability assessments. However, the use of aggregate size distribution as a means of soil structural analysis potentially allows changes at different scales to be investigated, particularly under controlled laboratory conditions. The breakdown of soil as a result of using this method will be solely dependent on the methods used to separate the soil, especially the level of energy applied to disperse the soil. It is therefore necessary to ensure that the disturbance will be comparable across investigations. The physical separation of the soil into various size fractions can mean that soil is assessed at scales, which may neither be applicable (for the associated biological processes) nor fully understood. The method does not assess soil by isolating processes responsible for changes in stability, such as changing pore dimensions, water relations and physical enmeshment of particles and it is not known how this measure may relate to water infiltration.

1.3.4 Soil water processes: Assessing infiltration of water into soil

Soil microorganisms are increasingly recognised to have a significant impact upon soil structure and water processes. Preston *et al.* (1999) showed the impact of native soil organisms on macro and meso-scale structural generation, and the allied effect of carbon substrate quality (Preston *et al.*, 2001). Baveye *et al.* (1998) observed the

impact of bacteria on water flow under high carbon loadings as a result of pore blocking. Vegetation and microbial activity can directly impact upon soil water infiltration by inducing levels of soil water repellency (Chan, 1992; Jaramillo *et al.*, 2000; McGhie and Posner, 1980; Miller and Wilkinson, 1977; Moore and Blackwell, 1998; Savage *et al.*, 1969; White *et al.*, 2000; York and Canaway, 2000). Changes to soil water repellency may result in changes to soil stabilisation and associated water processes, and hence means of analysis and the mechanisms involved have been researched quite comprehensively (Clothier and White, 1980; Hallett and Young, 1999; King, 1981; Letey, 1969; Tillman *et al.*, 1989; Van't Woundt, 1959). The role of microorganisms, in particular fungi, on reducing water infiltration rates forms a major part of this thesis, and will be discussed in more detail in Section 1.3.4.2. As background, a brief introduction is presented to several mechanisms involved in water infiltration into soil focussing on the development of hydrophobicity in soil systems.

1.3.4.1 Soil physics of water repellency

The process of soil water infiltration is controlled by a number of factors associated with soil and water properties these can be defined as:

Infiltrability: Is the amount of water per unit time a given soil profile can absorb through its surface when it is maintained in contact with water at atmospheric pressure.

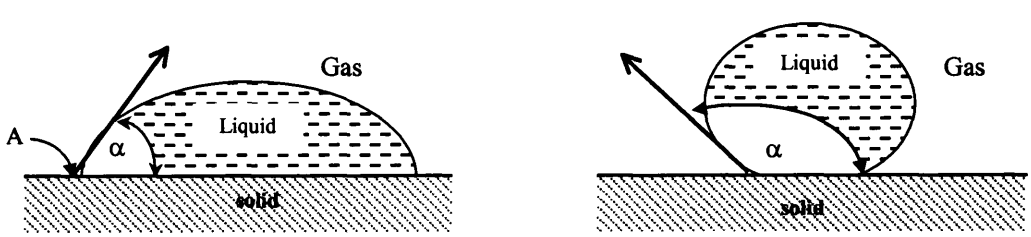
Wettability: The wettability of a solid such as soil particles is described by the contact angle between the wetting agent and the solid.

Sorptivity: Is the dominant factor responsible in the initial stages of infiltration and is dependent on the capillary properties of soil and decreases as the initial moisture increases (Hillel, 1998).

Philip (1957) measured sorptivity (S) by determining the slope of cumulative infiltration (I), which is the cumulative volume of water that has entered a set area of soil per unit of time (t), versus $t^{1/2}$ as shown:

$$I = St^{1/2} \tag{1.1}$$

Philip (1969) also reported a parameter known as intrinsic sorptivity, which takes into account the viscosity and surface tension of the liquid. A dominant factor involved in the infiltration of a liquid into a soil will be the level of contact between the soil and liquid. The spread of a liquid over a solid can be quantified by solid-liquid contact angle, and this will be related to the surface tension of the liquid. For instance, the attractive forces on a surface such as glass cause a droplet to spread out thinly and evenly on the surface, offering a small contact angle (often near to zero) [Fig 1.3]. In soil, the amphiphilic molecules from organic sources that coat mineral surfaces often reposition so that non-polar ends of the molecules face outward causing water molecules to adhere to one another rather than the soil surface resulting in a larger contact angle (Doerr *et al.*, 2000) [Fig 1.3 & 1.4]. Exposure to hydrophobic surfaces causes the adhesive forces between the solid and liquid to be greater than the liquid (Marshall and Holmes, 1979). The infiltration rate of water over time will increase as the contact angle decreases.



Hydrophilic surface Hydrophobic surface

Fig 1.3 Contact angles of a droplet upon a hydrophilic surface and a hydrophobic surface, demonstrating the differences in contact angle. The angle of the liquid is represented as: α, whilst "A" represents the point at which the three phases meet. Diagram adapted from Hillel (1998)

When dry, some soils exhibit hydrophobic properties that prevent the direct flow of water into the soil (Fig. 1.4). A droplet of water on a repellent soil surface may

remain for up to three hours, compared to a non-repellent soil where water would typically be absorbed within 0.1 seconds (King, 1981).

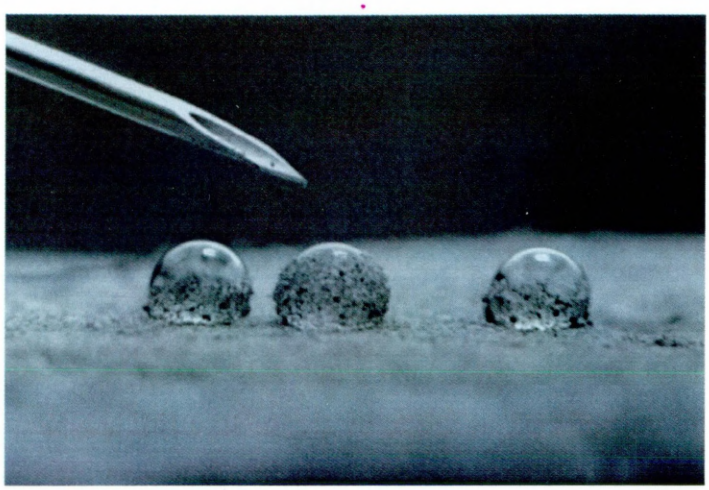


Fig. 1.4 Demonstration of extreme water repellency: water droplets on a soil surface resisting infiltration. Reprinted from Doerr *et al.* (2000), with permission from Elsevier.

Philip (1969) suggested that the majority of soils would wet readily and exhibit non-water repellent properties, with severe hydrophobicity considered an extreme property. Tillman *et al.* (1989) recognised that some soils that appear to wet readily when water is applied at either zero or negative pressure actually have a significantly reduced rate of wetting because of repellency. They termed this phenomenon as subcritical repellency. King (1981) did not distinguish between critical and subcritical repellency but classed repellency into four categories: non-significant, low, moderate and undefined/severe. Regardless of the classification of repellency, it directly influences water flow into soil even at very low levels (Tillman *et al.*, 1989; Wallis *et al.*, 1991). Repellency can be measured or classified in a number of ways based on quantifying the sorptivity or absorption of liquid into soil.

1.3.4.2 Causes of water repellency

Van't Woundt (1959) proposed that the phenomenon of repellency in soils was caused by calcium and magnesium soaps present in certain fertilizers. However, other authors have since reported that the effects are more likely to be microbial or plant residue related (Chan, 1992; Jaramillo *et al.*, 2000; McGhie and Posner, 1980;

Miller and Wilkinson, 1977; Moore and Blackwell, 1998; Savage *et al.*, 1969; White *et al.*, 2000; York and Canaway, 2000).

In some environments the vegetation type is responsible for inducing water repellency: plants that express hydrophobic mobile surface waxes directly affect water repellency. Vegetation known to such exude surface waxes include some evergreen trees including citrus (Richardson and Hole, 1978), eucalyptus (Doerr *et al.*, 1996; Ferreira *et al.*, 2000; Moore and Blackwell, 1998), and some species of pine tree (Moore and Blackwell, 1998; Richardson and Hole, 1978). It is thought that plants may exude hydrophobic substances as a means of suppressing the germination of competing vegetation (Moore and Blackwell, 1998). The organic matter associated with some of the species reported also has an effect upon soil water repellency as it tends to be complex and difficult to break down, resulting in a build up of hydrophobic organic material (Doerr *et al.*, 2000; Franco *et al.*, 2000).

Doerr *et al.* (2000) reviewed and reported that there are a number of hydrophobic substances produced by fungi, and bacteria, whilst Jex *et al.* (1985) reported that actinomycetes also affected water repellency. Fungi generally constitute two thirds of soil biomass (Denef *et al.*, 2001; Lyda, 1981) and are extensively reported to affect soil water repellency (Bond and Harris, 1964; Miller and Wilkinson, 1977; Savage *et al.*, 1969; White *et al.*, 2000; York and Canaway, 2000). However many of these investigations report fungal impacts along with contributions of fire induced changes to repellency. York and Canaway (2000) identified one of the causes of dry patches on golf greens to be the presence of basidiomycetes, with particularly high levels of water repellency detected in regions with large quantities of mycelia. York and Canaway (2000) also found evidence of high levels of water repellency in areas where the fungi had "passed through" indicating some sort of legacy effect either from (possibly unviable) mycelia or residual exudates.

Fungal hyphae can produce molecules such as hydrophobins and melanins, which may affect hyphal and soil hydrophobicity. Hydrophobins are secreted proteins produced by fungi, which are responsible for making hydrophobic surfaces hydrophilic and hydrophilic surfaces hydrophobic. These proteins are involved in

aerial hyphal growth, signalling and attachment of fungal hyphae to hydrophobic surfaces (Wösten and De Vocht, 2000). Melanins are secondary metabolites that are produced in large amounts after active growth has ceased. Melanins are deposited on the exterior surface of hyphae or other fungal structures and as a result fungi possess increased virulence and resistance to microbial attack, as well as enhanced survival while under environmental stress (Butler and Day, 1998; Smith and Berry, 1976). Due to the nature of these molecules both hydrophobins and melanins may be capable of changing the wettability of a surface and may therefore play a role in soil hydrophobicity (Wessels, 1996).

Soil hydrophobic compounds have been identified in plants and microorganisms. Doerr *et al.* (2000) reviewed some of these compounds, which include: *n*-alkanes, olefines, terpenoides, monoketones, β-diketones and polyesters of hydroxy-fatty acids. In contrast to this there are some naturally occurring hydrophilic substances produced naturally, such as rhamnolipids exuded by bacteria (Espinosa-Ugerl, 2003). The majority of biosurfactants can be classified into one of the following molecular groups; glycolipids, phospholipids and fatty acids and lipoproteins (Lang, 2002). Some of these hydrophilic substances have been cited in the bioremediation of polluted soils (Christofi and Ivshina, 2002) and will undoubtedly change the wetting properties of a soil.

Differing fungal species may produce a variety of substances which affect soil wetting and as a result contrasting reports exist of the impact of certain fungal species on repellency, with some researchers reporting increases in repellency whilst others note fungal induced reductions (Doerr *et al.*, 2000). It is possible that under certain conditions a particular species may increase repellency through a deposition of hydrophobic substances, whereas in other conditions it will consume hydrophobic substances and reduce repellency. The lack of understanding of particular species or interactions between microorganisms and other soil factors require further investigation.

The level of subcritical repellency in a soil will undoubtedly result from a combination of the various factors discussed, however the interactions between the

factors involved is not understood, particularly in agricultural soils where there is a high turnover of organic material and regular soil disruptions.

1.3.4.3 Water drop penetration time

The water drop penetration time (WDPT) test measures the length of time that water persists on the soil surface (see previous Fig. 1.4). It relies on a visual observation of the length of time required for the water to be absorbed into the soil. The result of the measurement is usually expressed in seconds (Letey, 1969; Van't Woundt, 1959).

Doerr *et al.* (2000) highlighted that authors using the WDPT test have different descriptions of what they consider to be hydrophilic or hydrophobic soils, with the low end of the scale varying from WDPTs of 1 to 5 and 60 s for a hydrophilic soil. This variation between investigations makes comparisons difficult. The WDPT test is commonly used on highly repellent soils. It fails to detect soils expressing low levels of water repellency, as water remains on the soil surface for a very short period of time. This measurement is also dependent on the quality of contact between the water droplet and soil surface.

1.3.4.4 Rate of water infiltration

Water repellency may be quantified by measuring the volume of water that infiltrates into the soil using a small ring infiltrometer (Clothier and White, 1980; King, 1981). This set-up involves embedding a tube into the flattened surface of the soil, with a set volume of water placed into the small ring infiltrometer. The measurement is the length of time for the ponded water to infiltrate into the soil. The resulting infiltration rate is usually expressed in millimetres per minute. Clothier and White (1980) developed a field-based set-up to measure soil water sorptivity. Forcing a cylindrical Perspex column into the soil, the portion of the tube above soil was filled with water and blocked to allow the measurements to be made under negative pressure, eliminating the effects of macropores (Clothier and White, 1980). Franzluebbers (2002) used soil water infiltration rates to assess the impact of soil aggregation and water infiltration, finding that the sieving of no-till soils resulted in more rapid infiltration in comparison to undisturbed or non-sieved soils.

Whilst this measurement is good for a rapid indication of soil water infiltration in the field, the level of disturbance may influence the measurement. The nature of the measurement restricts the wetting area to some extent, with a core forced into the soil restricting the wetting bulb. The outer edges of the infiltrometer may block pore networks, to an extent forcing the water into the soil through a path that may not necessarily have been the "natural" flow of water into the soil.

1.3.4.5 Molarity of ethanol droplet

Molarity of ethanol droplet test was first described by Letey (1969), and utilises the different properties of ethanol compared to water. Ethanol is a non-polar liquid and its different contact properties result in it being unaffected by hydrophobic materials within the soil. By mixing water with ethanol, a range of liquids with different contact angles are produced. The rate of infiltration of these liquids into soil is related to the contact angle between the liquid and soil. Letey (1969) found that hydrophobic coatings applied to sand had no impact upon pure ethanol infiltration, with ethanol lowering the liquid surface tension and infiltrating regardless of contact conditions. They proposed that ethanol had a contact angle of zero, which would result in perfect wetting (Letey, 1969).

King (1981) described a modification of the original molarity of ethanol droplet test method whereby droplets of ethanol are placed on to the soil surface, at an increasing molarity (at 0.2 M intervals). King (1981) described the quantification of repellency as "*the molarity of ethanol which penetrated the soil surface in 10 s*". The disadvantage of the molarity of ethanol droplet test is that no "real" water infiltration measurement is made, so the assessment of soil infiltration is based only on ethanol measurements.

1.3.4.4 Repellency index

Developing on the work of Letey (1969), who suggested that ethanol had a contact angle of zero, Tillman *et al.* (1989) used measurements of both water and ethanol

sorptivity to assess water repellency. They used ethanol as an intrinsic measure of repellency, proposing that the comparison of this measurement to water would give an indication of true soil repellency. The repellency index is reliant on differences in measures of water and ethanol; the relationship between sorptivity of water S_W and a 95% ethanol solution S_E of a non-repellent soil can be represented as:

$$S_W = \left[\frac{(\mu_E / \gamma_E)^{1/2}}{(\mu_W / \gamma_W)^{1/2}} \right] S_E \tag{1.2}$$

Equation 1.2 takes into account the effects of viscosity and surface tension of the two liquids, where μ_E and μ_W represent the viscosity of ethanol and water (0.0012 Nsm^{-2} and 0.0010 Nsm^{-2} respectively), whilst, γ_E and γ_W represent the surface tension of the two liquids (0.023 Nm^{-1} and 0.073 Nm^{-1} respectively at 20°C). Using the given values in Equation. 1.2, repellency (R) can be calculated as:

$$R = 1.95 \left(\frac{S_E}{S_W} \right) \tag{1.3}$$

With the constant value of 1.95 being the ratio of viscosity and surface tension for water and ethanol. Tillman *et al.* (1989) suggested that measurements be made at a negative pressure head preventing preferential flow into macropores, with this difference taken into account upon calculation of the repellency index (*RI*). Soils with a high repellency value restrict wetting allowing a slower infiltration of liquid into a soil. With this method Tillman *et al.* (1989) found that differences in sorptivity measurements were related to the medium and not to the infiltrating liquid, and suggested this method be used for measuring low level or subcritical water repellency.

Wallis *et al.* (1991) measured subcritical water repellency using the repellency index, finding this method to be a more sensitive measure of water repellency than either the WDPT or the molarity of ethanol droplet test. Wallis *et al.* (1991) suggested a repellency index was a more "*physically significant parameter*" for measures of sub-critical repellency, being a more sensitive measure than other

methods, and detecting differences between soils that other measurements may not have distinguished.

The repellency index method developed by Tillman *et al.* (1989) was scaled down to test individual soil aggregates and small soil volumes by Hallett and Young (1999). They accomplished this by using a small-scale infiltrometer device designed by Leeds-Harrison *et al.* (1994). Their device had an infiltrometer tip of a few millimetres diameter that was packed with sponge to allow for the establishment of a negative hydraulic head, which prevents the preferential flow of liquid into macropores. The tip was linked to a reservoir that had a horizontal capillary tube attached with a meniscus to monitor uptake of liquid by the soil (Leeds-Harrison *et al.*, 1994). They found this set-up to be as accurate as measures of ponded water infiltration using a small ring infiltrometer, and that the infiltration tip could be adjusted accordingly to suit the size of sample with no detriment to accuracy. This measure could be carried out at a fine scale and allowed a quantifiable measure to be made as opposed to a subjective visual observation. The steady rate of water flow into a soil (Q) from a circular ponded water source is calculated as:

$$Q = \frac{4bS^2r}{f} \tag{1.4}$$

In Equation 1.4, b is the soil-water diffusion function, which represents the water infiltration rate over the soil-wetting front; b can be in the range of $0.5 \leq b \leq \pi/4$, with White and Sully (1987) using 0.55 as an "average" The soil-wetting front, which accounts for the value 4 in the equation, is the spherical region where water infiltrates steadily in the initial time period. S is the sorptivity of water into the soil. The radius of the infiltrometer tip is represented by r, whilst f represents the fillable pore space in the soil (Leeds-Harrison and Youngs, 1997). From Equation 1.4 sorptivity can be calculated as:

$$S = \sqrt{\frac{Qf}{4br}} \tag{1.5}$$

The advantage of the methods presented by Leeds-Harrison and Youngs (1997) and Leeds-Harrison *et al.* (1994) is that an infiltrometer allows good soil contact that may not be achieved by other methods. By using ethanol in addition to water, Hallett and Young (1999) were able to assess the repellency using Equation 1.5. Measures of water infiltration or subcritical repellency allow the quantification of a soil process in the same manner as field based soil water infiltration.

1.3.5 Methods for visualising soil structure

Physical measures of soil structure are important to assess the behaviour of soil in conditions similar to those in the field, but often result in the disruption of soil. Visualising soil structures allows morphological changes in the soil to be observed in a natural state, at scales equivalent to the microorganisms and fine roots, which are thought to be central to any changes. This can be achieved using a number of well-developed and novel non-invasive soil tomography techniques.

1.3.5.1 Soil micromorphology using microscopy & thin sections

Thin sections are a relatively well-used technique that give a two-dimensional visualisation of soil structure, and more recently biology. Thin sectioning involves fixing the soil, by impregnating with resin, hardening and curing of samples, then sectioning of the samples into slices thin enough to view microscopically. The technique is relatively time-consuming, typically taking two weeks to get to the sectioning stage (Harris *et al.*, 2002). The majority of researchers using thin sectioning report a working resolution of 30 μm (Harris *et al.*, 2003). The method gives the potential, through serial sectioning, to allow a three-dimensional viewing of structure (Kapur *et al.*, 1997; Phillips and Fitzpatrick, 1999; Vogel, 1997; Vogel and Kretzschmar, 1996). Paired with image analysis techniques, thin sectioning allows the micromorphological investigation of soil (Terribile and Fitzpatrick, 1995). Vogel (1997) used serial sectioning to determine pore connectivity from field sampled soil. The advantage of using thin sections is that both physical and

biological properties of the soil may be viewed simultaneously if the correct resins and viewing light source are selected (Altemüller and Van Vliet-Lanoe, 1990; Tippkötter and Ritz, 1996; Tippkötter *et al.*, 1986). Nunan *et al.* (2003) demonstrated this through the investigation of pore space location and bacterial populations, completing an in-depth micro-scale investigation.

1.3.5.2 X-ray computer assisted tomography

Successful non-invasive soil structure work has involved the use of computer tomography (CT). CT is an imaging technique, where a number of transmission measurements of an X-ray beam are used to reconstruct a cross sectional image of the scanned object. Utilized in medicine for non-invasive investigations, the use of CT in soil is on a smaller scale and higher resolution, Table 1.1 illustrates the various scales of CT available.

Method	Observation scale	Resolution
Conventional	m	mm
High resolution	dm	100 μm
Ultra-high-resolution	cm	10 μm
Microtomography	**mm**	**μm**

Table 1.1 Methods of CT observations and the relative scales and resolutions achieved. Table based on Ketcham and Carlson (2001).

Unlike conventional medical CT X-ray scanners, which are metres in size and have a rotating X-ray source, X-ray microtomography typically has a fixed X-ray source with a large beam, panoramic 2-D detector and a rotating sample stage, with the set-up reduced to a scale of centimetres (Flannery *et al.*, 1987). Microtomography or high resolution CT is suitable for relatively small-scale soil structural analysis.

Microtomographical analysis of a sample involves a photon beam passing through a sample. At each image capture a detector measures how many photons have passed through a sample: the sample will attenuate photons to varying extents. The source energy and the density of the sample will dictate levels of attenuation, with the

density of the object generating the contrast due to differences in absorption. In the case of soil these differences will be between various particles, and differences between solid and pore space (Macedo *et al.*, 1999). As an X-ray beam passes through the sample in a number of different paths, each image taken is a two dimensional slice of the sample. The image thickness will be set prior to scanning and will be dependent on the particular conditions such as X-ray energy, beam thickness, and available memory for the resulting images. The output of CT scanning is a stack of cross sectional images, each of which may be individually or bulk analysed. This technique allows the observation of pore space, shape and connectivity and increases the number of digital images captured allowing for complex image analysis to take place which would have otherwise been time consuming and unfeasible.

Warner *et al.* (1989) used a combination of CT and dye staining to detect macropores, to a resolution of approximately 500 μm with image "slices" that were 8 mm in thickness. At a higher resolution, Macedo *et al.* (1999) used microtomography to characterise soil pore systems detecting differences at a resolution of 10 μm. A number of researchers have used CT analysis in order to determine the impact of water upon soil structure (Hainsworth and Aylmore, 1983; Heijs *et al.*, 1995; Phogat and Aylmore, 1989), with scale being the restrictive factor, limiting investigations of microbial impacts upon soil porosity and structure.

Limitations of microtomography upon small-scale soil samples to date have been the power and brilliance of the X-ray beam as soil has a relative high attenuation (in comparison to other biological materials). X-ray beam hardening can occur as the X-ray passes through the sample, with low energy X-rays attenuating more rapidly; the result of this is increased brightness at the outer edges of samples (Ketcham and Carlson, 2001). Increasing the potential of micro CT would involve the use of a more powerful X-ray source. Grodzins (1983) suggested that synchrotron light sources could be used for microtomographic imaging. Synchrotron light sources consist of a large storage ring of extremely high energy rapidly circulating electrons. As they circulate, the trajectory and velocity of electron flow is manipulated through a series of magnets. As the direction of the rapidly moving electrons is changed, photons are emitted, at X-ray wavelengths (Diamond, 2003). Third generation

synchrotron light source emits intense narrow beams of radiation, with 7 gigaelectronvolts (GeV) generated upon emission of synchrotron radiation, compared to an average of ≤125 kiloelectronvolts (KeV) generated by conventional medical X-ray sources (Ketcham and Carlson, 2001). Flannery *et al.* (1987) was one of the first to report the use of micro-tomography using a synchrotron light source to scan environmental samples of sandstone and coal, but to date the utilisation of this technology in soil structure investigations has been limited.

The application of high resolution X-ray microtomography allows researchers to visualise and quantify soil structures without excessive disruption of the soil. To date, however, little experimental work has been paired with this technology to assess how changes at the micro-scale impact upon the macro-scale properties.

1.3.5.3 Other methods of visually assessing soil structure

Electron microscopy is a useful tool for visualising small-scale soil structure and biology and gives the ability to achieve high-resolution (0.2 nm) two-dimensional images. Foster (1988, 1994) used transmission electron microscopy to assess how tillage affects the distribution of microorganisms and organic matter within the soil. Foster (1988) encountered technical problems with staining methodologies and utilised the techniques purely for biological visualisation rather than soil structural analysis. The advantage of this technique is the high-resolution images achieved allowing the visualisation of biological matter within soil structures that would normally be impossible to see. The limitations of the methodology include difficulties in quantifying soil structural components.

Nuclear magnetic resonance (NMR) microscopy is another methodology producing high resolution (50-100 nm) images for investigation of soil structure. Disadvantages associated with this method are difficulties with the signal to noise ratio as a result of paramagnetic material within the soil, also the nature of the final output makes quantifiable analysis difficult (Crestana and Posadas, 1998; Macedo *et al.*, 1999).

1.4 Biochemical assays of microorganisms

The literature review has so far the highlighted importance of microorganisms, particularly fungi, to the soil physical structural stability and water infiltration. Methods of quantifying the fungal population, biomass and dynamics are essential and will be discussed.

1.4.1 Ergosterol, a distinctive fungal sterol

Ergosterol (ergosta-5,7,22-trien-3β-ol) was discovered in the *Claviceps purpurea* (or Ergot) fungus and is the predominant sterol in most fungi (Hart and Brookes, 1996). It is almost exclusive to fungi and thus has been used as a fungal biomarker. The only non-fungal sources of ergosterol are some protozoa and some green algae, but these sources are not believed to interfere with fungal recognition, as Newell (1992) suggested that protozoan and microalgal mass would need to be considerable before it was necessary to correct for non-fungal sources of ergosterol.

Ergosterol is found in the phospholipid bilayer of the fungal cell membrane and is thought to play a role in a number of membrane-oriented processes, in particular controlling membrane permeability (Grunwald, 1971). Ergosterol has chemical and physical properties which make it easy to determine in soil extracts. It contains $\delta^{5,7}$ homoannular-diene double bonding, which is rarely found in plant sterols and creates a distinctive pattern of ultra violet (UV) absorption at 282 nm, which is different from other sterols found in the soil environment (Seitz *et al.*, 1979). The higher than "normal" absorption of fungal ergosterol means that co-extracted plant sterols are unlikely to interfere significantly with ergosterol quantification. Ergosterol also has a rapid turnover and will usually decompose after fungal death, thus it acts as an accurate biomarker of living fungal biomass. Because of these factors ergosterol is utilised as a fungal biomarker in soil and other environments (such a leaf litter).

Ergosterol is specific for fungi unlike other biomarkers such as adenosine triphosphate (ATP) and chitin. Some researchers report inconsistencies in the conversion factors for ergosterol to fungal biomass (for review see Ruzicka *et al.*,

2000), although this may be linked to the various isolation procedures available, which vary in time, efficiency and practicality; these will be reviewed in Section 1.4.1.2.

1.4.1.1 Concentrations of ergosterol from fungi

The quantity of ergosterol varies between fungal species. Variations between a small number of species are illustrated in Table 1.2.

Fungal species	Ergosterol content (mg g^{-1} dry weight)
Amanita rubescens[1]	13.5-17.6
Boletus griseus[1]	10.7-11.9
Ceococcum geophilum[1]	3.0-3.5
Hebeloma crustuliniforme[1]	2.8-6.8
Lactarius pubescens[1]	5.0
Paxillus involutus[1]	8.1-9.2
Rhizopogon sp.[1]	5.1-7.8
Phanerochaete chrysosporium[2]	5.1

Table 1.2 Variations in ergosterol concentration between fungal species, [1]Antibus and Sinsabaugh (1993), [2]Davis and Lamar (1992).

Most of the fungi displayed in Table 1.2 were maintained in cultures (and ergosterol was not isolated directly from soil). However the data illustrates the considerable difference in ergosterol content between and within species. The variation may be passed on to soils, with different soils having very different fungal population distributions. Antibus and Sinsabaugh (1993) reported that ergosterol levels were significantly lower in AM fungi compared to ectomycorrhiza. Very little research has been completed specifically for quantifying AM fungal biomass by means of ergosterol, as within the soil system it is difficult if not impossible to separate AM fungi from other soil fungi. Frey *et al.* (1994) used ergosterol to quantify intra and extra-radical hyphae in a clean system using *Glomus intraradices* and reported a good correlation between hyphal length and ergosterol. Frey *et al.* (1994) did however find relatively low levels of ergosterol at 0.0634 mg g^{-1} mycelial weight, calculating ergosterol to contribute only 0.006% of the mycelial dry weight reporting also low levels of mycelia.

There may be species based differences in ergosterol concentration; this biomarker does not give detailed information of fungal populations, but importantly gives an indication of live, and most likely actively growing and respiring fungi, and is a suitable biomarker for studying changes in fungal biomass.

1.4.1.2 Methods of ergosterol isolation

The ergosterol assay was first developed by Seitz *et al.* (1979) and involved initial extraction, sonication, partitioning and evaporation and then high performance liquid chromatography (HPLC) analysis (Ruzicka *et al.*, 1995). Grant and West (1986) developed a protocol based on that of Seitz *et al.* (1979). The Grant and West (1986) method is lengthy and complicated, involving large volumes of solvents (≅ 450 ml), many different steps, and a relatively large sample size (40-60 g). Ruzicka *et al.* (1995) reported an improved methodology which was quicker, more efficient and proved to be of no detriment to the concentration of ergosterol extracted.

Grant and West (1986) investigated the efficiency of the ergosterol isolation procedure and reported mean losses of 8% (of total extracted ergosterol). The same percentage loss during the extraction procedure was also found by Hart and Brookes (1996). These levels are considered reasonable for the type of assessment carried out. Other methods published include a microwave-assisted extraction (MAE) (Young, 1995), a supercritical fluid extraction (SFE) (Young and Games, 1993) and a number of variations of the original Seitz *et al.* (1979) protocol. Although improvements have been made, some extraction procedures are still time consuming limiting sample throughput. Improvements to the protocol could be made to benefit from the suitability of this bio-marker for assessing soil fungal biomass.

1.4.1.3 Ergosterol to fungal biomass conversion

The variability of the ergosterol to fungal biomass conversion has led some researchers to the conclusion that ergosterol is a more significant guide to the fungal membrane area rather than fungal biomass (Ruzicka *et al.*, 2000; Wallander *et al.*,

1997). Frostegard and Bååth (1996) found a close correlation between ergosterol and a principal fatty acid of fungal membranes. The majority of researchers have found good correlations between ergosterol and other indicators such as direct microscopy techniques (Frey *et al.*, 1992; Frey *et al.*, 1994; Montgomery *et al.*, 2000; Ruzicka *et al.*, 2000; Ruzicka *et al.*, 1995; Seitz *et al.*, 1979; Stahl and Parkin, 1996; Suberkropp *et al.*, 1993). One of the few negative aspects of ergosterol analysis is that the quantification of this sterol does not permit a differentiation between saprophytic and AM fungi. Methodologies that allow the quantification of separate fungal groups are discussed further in Section 1.4.3.

1.4.2 Glomalin, an exudate of arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are a group of soil fungi that develop a symbiotic relationship with plant roots; the arbuscule is a structure found in cells of roots where the transfer of nutrients occurs between fungi and roots, whilst "mycorrhizal" indicates the root orientated habitat (Wright and Morton, 1989). AM fungi play a role in the development of stable soil structure: the hyphae of the fungi attach to soil particles creating macroaggregates whilst exudates produced by AM fungi may also be responsible for enhancing soil structure. AM fungi have been shown to produce a substance called glomalin, first isolated by Wright and Upadhyaya (1996). Glomalin is a glycoprotein containing N-linked oligosaccharides (Wright *et al.*, 1998) and is excreted through the hyphae of AM fungi. This protein has been correlated to the stability of soil structure (Wright *et al.*, 1999).

1.4.2.1 Glomalin isolation and quantification

It is likely that glomalin remained undiscovered or un-extracted until recently because of the harsh conditions required to isolate this glycoprotein, which is insoluble in water extraction and requires heating a soil slurry with a citrate solution above 120°C (Wright and Upadhyaya, 1996). Wright and Upadhyaya developed a monoclonal antibody (MAb) that binds to glomalin (Wright *et al.*, 1996; Wright and

Upadhyaya, 1999). The antibody was initially raised against fresh spores of *Glomus intraradices* (FL208) and is known as Mab 32b11; it is specific to glomalin (Wright *et al.*, 1996; Wright and Upadhyaya, 1999). The antibody enables the visualisation of glomalin *in situ* on hyphae and soil surfaces through the addition of a fluorescently tagged antibody and subsequent observation under UV light (Fig. 1.5).

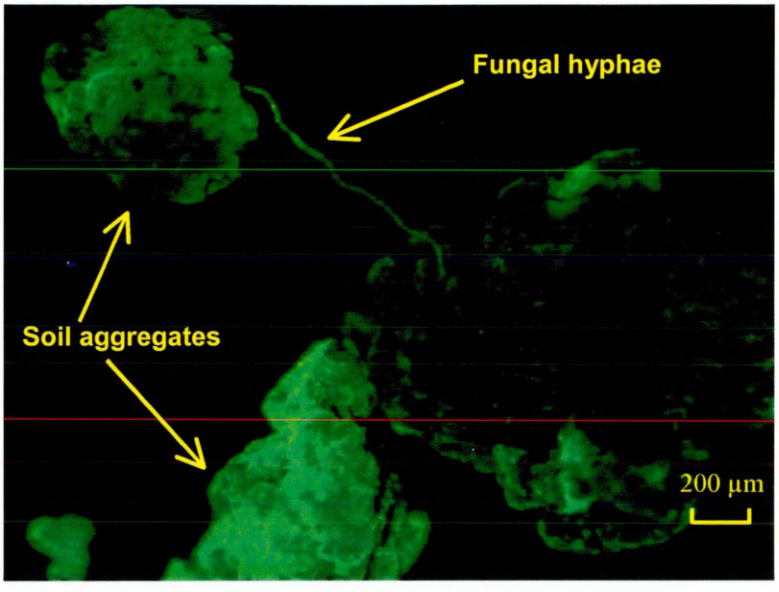


Fig. 1.5 A 1-2 mm soil aggregate viewed under UV light at × 50 magnification after incubation with both MAb 32b11 and a fluorescently tagged antibody (Image taken by D. Feeney at USDA Beltsville Agricultural Research Centre, MD, 2001).

The MAb can also be used indirectly on extracts allowing quantification using an enzyme-linked immunosorbent assay (ELISA) (Wright and Upadhyaya, 1998). Glomalin can also be measured through the use of the Bradford assay (Bradford, 1976; Wright *et al.*, 1999; Wright and Upadhyaya, 1996). Comparing the results of the indirect ELISA and the Bradford assay have revealed similar levels of glomalin, establishing that the antibody used in the ELISA is accurate for quantifying protein concentration. Quantifying total protein content of glomalin extracts through the Bradford assay assumes that the harsh extraction procedure denatures or destroys all other soil proteins. This assumption has been backed up by Rillig *et al.* (2001b) who demonstrated using nuclear magnetic resonance (NMR) spectroscopy that glomalin was the most abundant compound in the soil extract. Additionally, using sodium

dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) comparing extracts from pure cultures and soils, Rillig *et al.* (2001b) detected no additional bands in soil extracts, indicating that soil protein extracts contained only the protein of interest.

Wright and Upadhyaya (1998) reported that there are two different fractions of glomalin, labelling them as easily extractable glomalin (EEG) and total glomalin (TG). The total glomalin required more extreme conditions to extract including higher temperature and a higher concentration of citrate solution. Wright and Upadhyaya (1998) also reported that the EEG had a higher content of immunofluorescent material. These differences in immunofluorescence led Wright and Upadhyaya (1998) to further subdivide the existing two fractions, into immunoreactive easily extractable glomalin (IREEG) and immunoreactive total glomalin (IRTG). Different fractions may exist due to age of the protein and the resulting degradation of the antibody-binding sites (Wright and Upadhyaya, 1998). Additionally, the binding site may not always be easily accessible to the antibody and could be blocked by other components in the soil.

It is little understood for how long and why glomalin persists in soil. One estimate attempting to "date" glomalin has come from Rillig *et al.* (2001b) who measured the ^{14}C of glomalin and found the residence time of glomalin to be somewhere in the region of 6-42 years. This investigation however, was performed in forested tropical soil, and obviously conditions may be very different for other soils.

1.4.2.2 Concentrations of glomalin

Different species of fungi may vary in the concentration of glomalin produced (Miller and Jastrow, 2000). This is illustrated through results produced by Wright *et al.* (1996), shown in Table 1.3.

Culture	Yield $\mu g\ mg^{-1}$
Gigaspora gigantea MA453A	63
Gigaspora rosea UT102	60
Glomus etunicatum UT316	12
Glomus intraradices UT126	21
Glomus intraradices FL208	17

Table 1.3 Yields of immunoreactive protein extracted from pot cultures of AM hyphae, protein is expressed as total glomalin (μg) per mg hyphae (values taken from Wright *et al.*, 1996).

The results displayed in Table 1.3 show that the total yield ($\mu g\ mg^{-1}$) could vary quite considerably across fungal species; the differences in quantities exuded are to date unexplained. A consideration to make when studying glomalin is that different soil types may have different fungal populations within them. For example, an arable site will have a completely different fungal population profile when compared to a woodland site (Helgason *et al.*, 1998). The profile in either soil could consist of AM fungi that do not produce particularly large quantities of glomalin. The resulting glomalin content could have less to do with the land management practice and more to do with the plants in the soil, with some AM fungal species showing host preference (Vandenkoornhuyse *et al.*, 2002).

The age and quality of the fungal population may also have a significant bearing on the quantity and volume of glomalin isolated from a soil sample, with immunofluorescent material more visible on "younger thinner and intact hyphae" than older and thicker hyphae (Wright *et al.*, 1996). However, this may not reflect the total glomalin content, and the lack of visible immunofluorescent material on older hyphae could indicate degradation of the antibody-binding site. The binding site on older hyphae may also be inaccessible having been covered with resistant organic matter or hydrophobins exuded by hyphae.

When first discovered, glomalin was suggested as a biomarker of arbuscular mycorrhizal fungi (Wright and Upadhyaya, 1996). More recent publications with conflicting results may give cause for debate over this use of glomalin, with varying reports of the relationship between AM hyphal length and glomalin concentration.

Discrepancies reported by Steinberg and Rillig (2003) and Rillig *et al.* (2001a) between protein concentration and hyphal length measurements highlight the caution that should be taken when using glomalin as a potential AM fungal bio-marker.

1.4.2.3 Structural composition

The biosynthesis and chemical structure of glomalin to date remains relatively poorly understood, although work is currently ongoing. Wright and Upadhyaya (1998) suggested that iron might somehow be associated with glomalin, giving the extracts a distinctive red-brown colouration. They found 0.8-8.8% iron in some extracts and suggested that low glomalin concentrations may somehow be related to soil iron deficiencies. Rillig *et al.* (2001b) also presented data highlighting a possible link with iron. Using NMR spectroscopy, they attempted to establish the structural components of the substance, finding peaks that indicated key structural features such as a peptide backbone and aromatic amino acids, and the possibility of peptide methine chains. This is however, as far as the structural understanding of this substance has progressed.

Glomalin may be one of a range of persistent proteins in soil that play a role in the stability of soil structure, having been positively correlated with measures of aggregate stability (Franzluebbers *et al.*, 2000; Rillig *et al.*, 2002; Wright and Anderson, 2000; Wright *et al.*, 1999; Wright and Upadhyaya, 1998). It is thought to prevent excess wetting of aggregates thus causing increased stability (Miller and Jastrow, 2000), but the mode of action of glomalin remains untested and is purely hypothetical. Only one physical assessment (aggregate stability) of soil structure has been associated with this protein (Wright and Upadhyaya, 1998). This is an area of research that requires further investigation.

1.4.3 Other methods of microbial quantification

Whilst both ergosterol and glomalin give an indication of the fungal community size, neither method measures the wider microbial community. Therefore other methodologies will be briefly discussed.

1.4.3.1 Phospholipid fatty acid analysis

Ester linked fatty acids in phospholipids are one of the most sensitive methods of chemically analysing microbial community structure (Guckert and White, 1986). Phospholipid ester-linked fatty acids (PLFA) are essential components of the membranes of all cells, and play a major role in maintaining cell structure. Once extracted from environmental samples such as soils or sediments, lipids can be analysed by gas chromatography or mass spectrometry allowing the identification of individual lipids. A profile of fatty acids allows the characterisation of microbial communities. Microbial groups can be identified through specific "*signature*" phospholipid fatty acids (Tunlid and White, 1992), which are unusual lipid patterns that are specific to certain microbial groups, such as fungi, which synthesise saturated even-chained polyenoic fatty acids (Tunlid and White, 1992). Some PLFA signatures for soil organisms are shown in Table 1.4.

PLFA Signatures	Organism
16:1ω9, i15:0	Eubacteria, cyanobacteria, actinomycetes
16:0, 18:3ω6	Fungi
16:1ω5	Cyanobacteria, AM fungi
16:1ω7, 16:1ω7t	Eubacterial aerobes
18:1ω7	Eubacterial aerobes, Gram negative bacteria
18:2ω6	Eukaryotes, cyanobacteria, fungi
18:3ω3, 18:ω6	Fungi, green algae, higher plants
20:3ω6, 20:4ω6	Protozoa

Table 1.4 Signature phospholipid fatty acids [Data taken from Paul and Clark (1996)].

The PLFA signatures shown in Table 1.4 are expressed as the total number of carbon atoms followed by the number of double bonds with the position closest to the aliphatic (ω) end, whilst the prefix "i" refers to "iso" (Tunlid and White, 1992). Phospholipids are isolated from soil through a protocol developed by Bligh and Dyer (1959) and the extraction process for 15-20 samples can be completed in approximately 2 days (Tunlid and White, 1992). Analysis of PLFA allows the investigation of microbial community profiles and estimation of biomass of environmental samples (Bardgett and McAlister, 1999; Frostegard and Bååth, 1996; Frostegard *et al.*, 1996; Zelles *et al.*, 1992).

Analysis of the signature PLFA's allows for an accurate assessment of specific microbial groups. Furthermore total PLFA analysis allows a quantitative measurement of viable biomass. The analysis gives a viable biomass measurement, as the cell membrane of microbes must be intact for phospholipids to be present. An advantage of this technique highlighted by Frostegard and Bååth (1996) is that the same method can be used to assess both bacterial and fungal biomass.

1.4.3.2 Adenosine triphosphate

Adenosine triphosphate (ATP) is a cellular component quantified by the luciferase assay. Subject to rapid decomposition within the soil, ATP is considered to be a good indicator of microbial biomass. The shortcomings of using ATP include the variety in extraction procedures, harsh acidic extractions prevent any ATPase activity, therefore more neutral reagents often detect much lower levels of ATP making comparisons between various results difficult (Powlson, 1994). Another possible downfall of using ATP is that it is not specific for any microbial group, being present in bacteria, fungi, protozoa, algae, actinomycetes and plants tissue, giving an indication of total biomass. ATP may also be detected from dead cells (Tunlid and White, 1992).

1.4.3.3 Chitin

Chitin is a major component of the fungal cell wall and can be used to quantify fungal biomass. A polymer of N-acetylglucosamine, after hydrolysis chitin produces glucosamine, which can then be quantified either spectrophotometrically, or though HPLC analysis (Tunlid and White, 1992). The downfalls of using chitin include the fact that it is not specific for living fungi (Frey *et al.*, 1994), and there is often interference of native soil amino acids (Johnson and McGill, 1990). Chitin has also been detected in other soil organisms [e.g. bacteria and invertebrates (Tunlid and White, 1992)].

1.4.3.4 Microscopy/hyphal counts

Alongside chemical indicators of fungal biomass, some researches still opt for traditional microscopy, using measures of hyphal length as indication of fungal biomass or cell counts for bacteria. Fungal hyphae can be separated from the soil by sieving techniques and quantified by the gridline intersect method (Miller *et al.*, 1995; Olson, 1950). AM fungi can be separated from saprophytic fungi through the positive recognition of AM fungal morphological features, this however is at risk of operator difficulties and is a factor that is considered to be one of the problems associated with the investigation of soil fungi (Kunc, 1994). Image analysis techniques may be used to quantify fungal hyphae in soil, and allow for increased throughput of samples with some steps being computer automated (Harris *et al.*, 2003). The introduction of automated steps may also reduce operator effects.

1.4.3.5 Microbial respiration

The process of respiration by soil microorganisms involves the oxidisation of organic matter; the end products of this reaction are CO_2 and water. The metabolic activity of a soil microbial population can therefore be quantified through CO_2 production or O_2 consumption. Basal respiration can be defined as respiration with no organic substrate additions, whilst substrate induced respiration (SIR) is the measure of soil respiration in the presence of an added substrate such as glucose (Alef, 1995). Sparling (1995) suggests that SIR makes a number of assumptions; the

various organisms in the soil will respond in a constant manner to SIR and during the test period the majority of soil organisms will respond. The substrate added is suitable in order to achieve maximal respiration, and finally the contributions from non-glucose metabolising organisms are minimal. Measures of respiration are responsive to changes in soil such as moisture content and temperature, making this a sensitive method of assessment.

Respiration measurements can be coupled with selective inhibition using antibiotics such as cycloheximide and streptomycin, establishing the contribution of either fungi or bacteria to total respiration (Lin and Brookes, 1999a,b; Velvis, 1997). Disadvantages associated with microbial inhibitors include: microbial inactivation of the inhibitor, non-target effects, and variations in the ratio of sensitive and insensitive organisms, which may differ depending on the soil type (Nannipieri *et al.*, 1994; Velvis, 1997).

1.4.3.6 Molecular analysis

The application of molecular techniques allows both quantitative and qualitative investigation of the genetic diversity of soil organisms. The isolation of DNA and RNA from soils permits the application of gene probes and various molecular techniques such as, PCR (ratio and function analysis), molecular finger printing (using DGGE and TGGE) and fluorescent *in situ* hybridisation (FISH) to investigate various aspects of the microbial community. These methods potentially allow the analysis of both structure and function of microbial communities (Kunc, 1994). The analysis of soil microbial populations using molecular analysis allows a highly specific investigation that may not be necessary for investigations of total biomass.

1.5 Practical significance of biophysical interactions upon soil sustainability

Both physical and nutritional changes to the soil system will have direct and indirect consequences for both soil structure and microbial make-up.

1.5.1 Impact of disruption and land management

"*Tillage involves the mechanical manipulation of the soil with the objective of promoting good tilth, and, in turn, higher crop production*" (Brady, 1974). Conventional tillage cuts up and incorporates crop residues into the soil, thus allowing the crop residues to be decomposed rapidly. Immediately after ploughing the soil is loosened creating an increase in total pore space and the creation of a weed-free environment for planting. The long-term effects of tillage include the breakdown of organic residues, causing a reduction in the soil organic matter (SOM) content, and disruption to microbial communities, particularly those associated with stabilisation of soil.

The physical disruption of soil through the process of tillage is known to affect microbial populations and processes (Doran, 1980; Lupwayi *et al.*, 1998; Young and Ritz, 2000). Tillage generally results in a reduction in the biological activity of soils in comparison to a zero tillage or undisturbed soil systems. The effects of tillage upon the microbial community will be dependent on the scale of the microbes involved. Bacteria may remain intact as they are smaller in comparison to fungi, where mycelia will be more prone to disruption (Young and Ritz, 2000). The disruption of fungal hyphae was reported by both Beare *et al.* (1992) and Roberson *et al.* (1995), who proposed that the importance of fungi in soil structure and function was greater in reduced or no-tillage systems.

Doran (1980) and Lupwayi *et al.* (1998) investigated tillage and crop sequence effects upon microbial functional diversity and community structure. Both researchers compared conventional and zero tillage systems. They found that tillage

caused reductions in microbial diversity. Doran (1980) reported that the largest differences were found in fungal populations, which were 57% greater in no-till than conventional tilled soil. Lupwayi *et al.* (1998) concluded that microbial diversity was reduced through tillage because of mechanical destruction (this effect would be more pronounced in fungal populations that bacterial), desiccation, and disruption of soil pore structures, which was also reported by Doran (1980). Large differences in fungal populations between tilled and no-tilled soil illustrates how fungi dominate the undisturbed soil structure.

The effect of disturbance upon fungal biomass can be clearly observed when various land management effects are compared. Using ergosterol as an indicator of fungal biomass, this comparison was made by a number of authors. Stahl and Parkin (1996) compared a number of different soil conditions and found the lowest mean ergosterol content in a conventional tillage corn-soybean field and the highest in a prairie site (Table 1.5). Djajakirana *et al.* (1996) also compared different soil types using ergosterol concentration as a measure of fungal biomass. Ergosterol concentrations were generally higher in grassland than arable sites with, on average, a two to threefold difference between arable and grassland ergosterol contents (Table 1.5).

Arable	Grassland/ Pasture/ Prairie	Forest	Comments	Publication
2.14	5.52	5.45	Forest: deciduous. Grassland sampled.	Djajakirana *et al.* (1996)
0.99	1.30-2.06	N/A	Grazed pastures sampled. Arable sample cultivated for barley. No forest samples taken.	Grant & West (1986)
1.03	3.49	2.36	Prairie sampled	Stahl & Parkin (1996)

Table 1.5 Mean ergosterol concentrations ($\mu g\ g^{-1}$ soil) under different land use conditions from a number of different publications. Data taken from Djajakirana *et al.*, (1996), Grant and West (1986), Stahl and Parkin (1996).

Differences in land practices, particularly the influence of disruption, will influence ergosterol concentrations. Tillage in particular is associated with reduced fungal biomass (Stahl and Parkin, 1996). Changes in fungal biomass are associated with

reductions in soil structural stability. Little work has, however, associated the importance of fungal populations in relation to soil water processes and additionally how these processes are related to typical structural assessments.

The effect of tillage upon the soil microbial community can have a considerable impact upon crop production as a result of the impact on fungal pathogens. Peters *et al.* (2003) detected reduced levels of infection by *Rhizoctonia solani* under reduced tillage systems proposing that these systems encouraged the antibiotic abilities of endophytic and root zone bacteria. James *et al.* (1997) found that the use of conservation tillage resulted in slower growth of seedlings compared to traditionally tilled soils. James *et al.* (1997) demonstrated that disturbing the soil positively enhanced shoot growth and reported these differences were due to a reduction in pathogens. Reduced tillage systems often leave crop residues in the soil allowing the maintenance of pathogens; considerations for specific crop rotations must be made when this method is used (Bockus and Shroyer, 1998).

1.5.2 Nutritional/chemical soil management

In addition to physical manipulations made to the soil, chemical amendments are also made to enhance the soil for increased productivity, another factor that influences soil microbial populations. Donnison *et al.* (2000) investigated changing fungal populations in hay meadow litter, assessing whether changes were due to the composition of litter type or the addition of fertilizers. Six fungal isolates, which originated from soil and litter, were selected for the investigation and assessed for the effects of high and low doses of NPK fertilizer. Donnison *et al.* (2000) showed that fertilizer addition affected each fungal species differently. This species-specific reaction increased mycelial growth rates in some species but induced inhibited growth in others. Donnison *et al.* (2000) demonstrated the ability of some fungal isolates to thrive in conditions of increased NPK, showing that the fungal community structure could be altered through the application of inorganic fertilization.

The addition of ammonium nitrate and urea causes decreases and increases in soil pH respectively (Wild, 1993), a factor also reported by Thirukkumaran and

Parkinson (2000), who reported that the application of ammonium nitrate caused significant decreases in basal respiration. They suggested that the maximal suppression in microbial respiration was linked with the most considerable change in pH. Mötönen *et al.* (1999) reported that soil fungal biomass had a high degree of spatial variability; this variation was correlated with pH, organic layer thickness and total C, K, Mg.

The relationship between chemically induced changes in pH and subsequent changes in microbial populations shows the fine balance in equilibrium maintained in soil. Different management practices involve the application of chemical and organic supplements to the soil, these affect soil microbiology considerably but it is little understood how these changes affect soil water processes.

1.6 Aims of Thesis

The overall aim of this research is to examine the interactions between fungi and the associated physical habitat. Specifically it will examine in detail

- The proposed effects of the AM fungal protein, glomalin, on soil water infiltration.
- The individual and combined effects of roots and fungi, in order to measure meso-scale (mm-cm) relationships between aggregate formation and stabilisation, fungi and factors related to soil water infiltration.
- Three-dimensional micro-scale (μm-mm) pore changes with respect to fungal and root impacts.
- The impact of land management on fungal biomass and soil water processes.

Chapter 2: A spatial examination of plant root and glomalin influences upon soil water infiltration

2.1 Introduction

Plants and fungi are known to enhance the physical stability of soil (Miller and Jastrow, 1990, 1992, 2000; Tisdall and Oades, 1982), but few studies have isolated or quantified the underlying mechanisms. One proposed mechanism is the physical enmeshment of soil particles, which stabilises soil by a 'stringy bag effect' (Jastrow *et al.*, 1998; Miller and Jastrow, 2000; Oades and Waters, 1991). Another mechanism is the secretion by fungi and roots of extra-cellular exudates that bind soil particles, thereby increasing mechanical resistance to disruption (Chenu and Guerif, 1991; Czarnes *et al.*, 2000; Roberson *et al.*, 1995). It has also been proposed that extra-cellular exudates enhance soil stability by changing the wetting properties of soil (Capriel, 1997). However, the specific compounds and organisms involved have not been isolated and few studies have examined the physical process of reduced wetting in detail.

Discussed comprehensively in Section 1.4.2, one exudate proposed to influence soil structure is glomalin (Wright *et al.*, 1996; Wright and Upadhyaya, 1996). It has been reported that the secretion of glomalin, a glycoprotein exuded by AM fungi, acts directly to increase soil aggregate stability by increases to hydrophobicity, thus significantly reducing the disruption of soil structure by slaking processes (Wright and Anderson, 2000; Wright *et al.*, 1996 & 1998; Wright and Upadhyaya, 1996, 1998 & 1999). Slaking occurs at the onset of wetting, when rapidly advancing water in soil pores creates a build-up of air pressure disrupting the soil's physical structure. As slaking may be responsible for the breakdown of structurally unstable soils, the increased stability provided by glomalin should, hypothetically, cause soil surfaces to become less permeable to water, which in addition to binding soil particles should reduce the risk of slaking. This mechanism, however, has not been validated and no direct *in situ* measurements of glomalin and water infiltration have been carried out.

The onset of water infiltration is physically described as water sorptivity, which is defined as the ability of soil to imbibe water, much like a sponge at the early stages of infiltration (Philip, 1957). Hallett and Young (1999) developed a new method of accurately measuring sorptivity at high resolution (< 10 mm), and by measuring both water and ethanol sorptivity, were able to quantify low levels of water repellency.

These data allow for the calculation of a repellency index, *R*, which has a parametric value directly proportional to the reduction in water sorptivity caused by hydrophobic processes (Hallett and Young, 1999). For instance, a soil with an *R* of 3 indicates that the rate of water infiltration, measured as sorptivity, would be 3× faster if the effects of hydrophobicity were removed. A direct measurement of water infiltration linked to glomalin quantification may allow for an accurate assessment of the glomalin/water infiltration relationship.

The aim of this chapter is to quantify the hypothesised influence of glomalin upon water infiltration. Glomalin levels are manipulated in a laboratory study by growing pea (*Pisum sativum,* cultivar: Fotune) plants in an arable soil with initially low glomalin levels. The change in glomalin concentration and repellency is assessed spatially within the soil pot to determine if any relationship exists between them. These data are compared to an unplanted control sample, where freshly exudated glomalin will not be present. The spatial heterogeneity of glomalin and repellency is also assessed, to determine the potential implications to the spatial complexity of soil physical structure. This work may provide the first quantitative evidence that soil stabilisation by repellency is directly linked to glomalin.

2.2 Materials and Methods

2.2.1 Soil core preparation

The soil used was an experimental arable field site, Bullion Field, situated at the Scottish Crop Research Institute. The soil is derived from undifferentiated sandstone (Carpow Series) and is comprised of 71% sand, 19% silt and 10% clay with a pH (H_2O) of 6.2, 1.9% C and 0.07% N (White *et al.*, 2000). It was sampled from the top 100 mm of a fallow plot and subsequently sieved to 2 mm. Soil was stored (overnight) at 4°C between sieving and packing to minimise biological activity.

The soil was packed into plastic cores with a diameter of 64 mm and height of 50 mm. Prior to packing, cores were split vertically and then taped securely to increase

the ease of later sampling. The initial gravimetric moisture content was measured (16.8% ± 0.05) and the soil packed into cores to a dry bulk density of 1.4 Mg m^{-3}.

Planted cores were prepared by placing a single pea seed, *Pisum sativum* (cultivar: Fotune) centrally within the top 10 mm of the soil core during packing. The base of the soil cores was covered with a fine mesh to contain the core contents but allow for the uptake of water. The soil surface was covered with a thin layer of black PVC beads to minimise evaporation. In total, five control cores containing only soil, and five planted cores each containing one pea seed, were used.

Cores were maintained in a growth cabinet 20°C, 80% humidity and 16 hours of light 8 hours dark per day (Fitotron: 40, 000 LUX, 500 mmol $m^{-2}s^{-1}$, 110 W m^{-2}). The incubatory period was 34 days based on the work of Olsson and Wilhelmsson (2000) who reported optimal AM fungal growth prior to 35 days incubation. During the incubatory period, the soil cores were watered to weight (maintaining cores at a constant 20% water content). In addition, cores were lightly misted daily with water to reduce drying of the soil surfaces.

2.2.2 Sampling regime

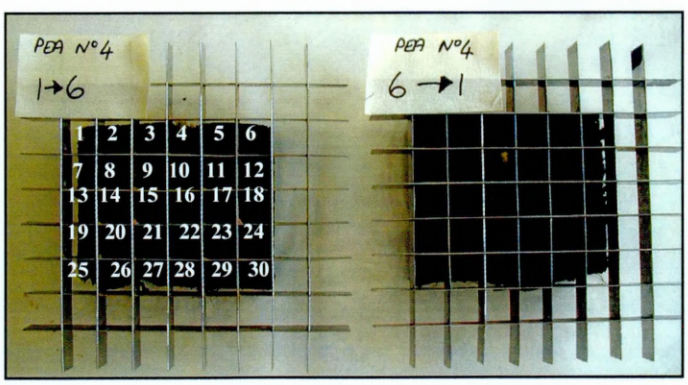
Cores were disassembled as illustrated in Fig. 2.1 and sampled to obtain two replicate spatial grids each containing 30 blocks of soil. Plant shoots and leaves were removed by cutting at the base of the stem. Soil cores were halved using a palette knife to cut along the vertical split in the soil container. This allowed for exposure of the central soil region with minimal disturbance. The exposed faces provided 2 spatially similar samples for subsequent analysis.

A metal grid, consisting of 10 × 10 mm cutting edges, was placed on the face of each soil portion and gently pushed into the soil. This resulted in thirty paired spatial soil samples, 1000 mm^3 in volume, taken from each core half. Soil samples were removed from the sampling grid with care taken to minimise disturbance upon removal soil sub-samples were treated independently of one another with no potential flux of materials between adjacent units of soil; one half of the core was

used for quantification of glomalin concentration whilst the opposing half was assessed for water repellency measurements.

Planted and unplanted cores removed from growth cabinet after 34 days incubation.

Shoots and leaves of plants removed. Remaining soil cores split in half to allow paired soil samples to be taken.



10 mm deep soil portion removed from each core half, and metal grid forced into soil giving thirty sub-samples.

Fig. 2.1 Sampling regime to establish paired spatial samples

2.2.3 Glomalin isolation

Glomalin concentration was measured in 30 spatial sub-samples per replicate core, using the method described by Wright *et al.* (1996). The soil sub-samples were first homogenised by breaking and mixing with a spatula. Each sub-sample was weighed and 50 mmol l^{-1} sodium citrate (pH 8.0 adjusted with HCl) was added at a volume of 8 ml per gram of fresh soil. The soil slurry was autoclaved at 121°C for one hour and then centrifuged at 2300 × g for 20 min, after which time the supernatant was removed and stored at 4°C.

A Bradford Protein assay (Bradford, 1976), (Pierce, Illinois, USA) was used to quantify the total protein content of the supernatant. Bovine serum albumin (BSA) was diluted with sodium citrate (as detailed above) to create a range of protein concentrations (25-2000 µg/ml) to create a standard curve. Standards and samples were mixed with the protein assay reagent at a ratio of 25 µl sample to 1.25 ml reagent. The liquids were mixed thoroughly in 2.5 ml cuvettes (Kartell) and left for ten minutes at room temperature prior to measurement. Using sodium citrate as a blank the absorbance of both standards and samples were measured using a spectrophotometer set to 595 nm (Thermo Spectronic, Genesys 10 UV). The absorbances of the standards were plotted and samples concentrations were established through extrapolation using the equation of the standard curve.

This method was deemed to quantify the protein concentration as accurately as the antibody specific ELISA (see section 1.4.2.1). The total extracts were expressed as milligram protein per gram of dry soil.

2.2.4 Water repellency

Soil water infiltration and repellency was measured on intact soil cubes removed from the sampling grid. Each cube was dried at 40°C for two days, thus providing a condition close to the maximum potential water repellency as defined by Dekker *et al.* (1998). Using a small-sized soil infiltrometer (1.75 mm in diameter), developed

by Hallett and Young (1999) and modified by White *et al.* (2000), soil water sorptivity and repellency were determined.

The apparatus (shown in Fig. 2.2) consists of a liquid reservoir, linked to an infiltrometer loosely packed with a sponge at its tip. The infiltrometer tip was held at a negative pressure head to the reservoir (-20 mm) to minimise macropore flow. Soil samples were raised into position so that the sponge tip of the infiltrometer had good soil contact. The loss of liquid from a reservoir placed upon a balance (±0.1 mg) was measured over a period of two minutes and recorded every ten seconds, until the water loss was consistent over each time period. Water sorptivity was measured first after which the sample was dried again at 40°C overnight so that ethanol sorptivity could be measured in the same manner. Equations 1.2-1.5 (Section 1.3.4.4) were used to calculate sorptivity from the flow rate of the liquid.

Water repellency (R) was determined using the approach of Tillman *et al.* (1989) based on the sorptivity of water, S_W and ethanol, S_E,

$$R = 1.95\left(\frac{S_E}{S_W}\right). \tag{2.1}$$

A number of factors may cause experimental error in the readings using this approach, but they were minimised by taking care during measurements. The sponge tip was washed between samples by dipping into a beaker that contained the infiltrating liquid (i.e. water or ethanol). Evaporation was reduced by using a reservoir container with a neck small enough to contain the piping with minimal surrounding space. The rate of evaporation, determined by leaving the apparatus for 5 minutes, was less than 1% for both liquids and therefore not significant. Liquid flow through the sponge was assessed regularly by simply lowering the tip below the reservoir level to check for liquid flow through the tip. There was consistent dripping of liquid, much more rapid than the rate of infiltration into soil, if the tip was functioning effectively. If this was not the case the sponge tip was cleaned or

repacked. The efficacy of the small-scale infiltrometer is described in detail by Leeds-Harrison *et al.* (1994), who report experimental error that is comparable to much larger infiltrometer designs.

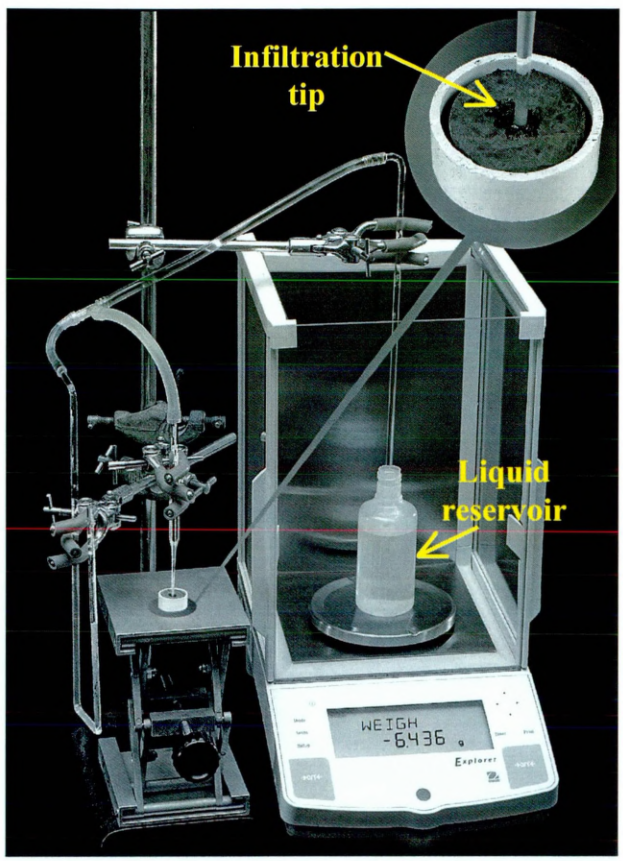


Fig. 2.2 Water repellency measurement set-up to quantify sorptivity and hydraulic conductivity of soil samples. Showing the balance and reservoir in relation to the infiltrometer, which is at a negative pressure, set-up based on Hallett and Young (1999).

2.2.5 Root staining for mycorrhizal colonization

AM colonization of roots was confirmed by staining. This confirmed whether AM fungi grew in the soil cores and therefore could potentially influence changes in glomalin concentrations. A single planted core was spatially sampled for root fragments (as shown in Fig. 2.1), which were then stained for AM hyphal colonization.

Roots were removed from the spatially sampled soil using fine forceps. The remaining soil was then washed through nylon mesh to ensure that all root fragments were removed from the soil sample. Root fragments were stained using an adaptation of the method reported by Phillips and Hayman (1970): samples were placed into 2 ml centrifuge tubes and washed thoroughly with tap water to remove any attached soil particles. Roots were cleared using 10% potassium hydroxide in a 90°C water bath for 7 minutes.

Immediately after clearing, samples were washed with copious amounts of tap water to remove the potassium hydroxide. Root samples were acidified by immersing in a 1% hydrochloric acid solution for one hour and stained using a 0.05% trypan blue solution (in lactoglycerol solution) in a 90°C water bath for 10 minutes. The trypan blue stain was removed with lactoglycerol (destain solution; lactic acid:glycerol:water at 14:1:1) overnight or longer if required, with longer destaining required for thicker root material. Times used throughout the staining process allowed adequate clearing and staining of samples whilst still maintaining root structure. Treatment times were optimised beforehand on trial samples subjected to a range of clearing and staining times.

Once thoroughly destained, root fragments were mounted horizontally in lactoglycerol solution on a microscope slide to observe for mycorrhizal colonization.

2.2.5.1 Quantification of mycorrhizal colonization

In order to quantify the percentage root length colonization (% RLC), mounted root samples were observed under a microscope at 100× magnification, and quantified using a crosshatched eyepiece graticule.

Roots were initially observed for arbuscular mycorrhizal structures such as vesicles and arbuscules. Once the presence of these was noted, the visible counted hyphae could be recorded as arbuscular mycorrhizal.

Roots samples were brought into the field of vision and quantified with an eyepiece graticule using the method of McGonigle *et al.* (1990). The intersections of the eyepiece graticule were examined for the presence of roots and mycorrhizas, with the number of intersections counted. Upon completion of the counts, the percentage root length colonized was calculated by dividing the number of intersects with arbuscular mycorrhizal fungi by the total intersects counted.

2.2.6 Statistical analysis

Both water repellency and glomalin data sets were subjected to an Anderson Darling normality test. The results of the normality test showed that both data sets were highly skewed ($P<0.01$), requiring a Box-Cox transformation to obtain a normal distribution ($P>0.05$). Transformed data was used for analysis thereafter.

The aim was to detect for any significant dependency between water repellency and glomalin concentration, and to quantify spatial dependencies in each separate treatment (i.e. either planted or unplanted). Differences between planted and unplanted conditions were analysed using a two-sample t-test, whilst the relationship between glomalin and water repellency was analysed using a Spearman rank correlation. In order to detect spatial differences between samples, a neighbour related analysis was used, which analysed data points in proximity to the plant (the same reference point was used in control cores). The data sets were assessed for autocorrelation using Moran's I statistic, which indicated spatial dependency in both glomalin and water repellency data sets ($P<0.001$). The spatial dependency was taken into account, and a multiple spatial regression analysis was applied to the glomalin and water repellency data sets.

2.3 Results

After 34 days of incubation, plants grew unsupported to a height of approximately 150 mm, with an average mass of 30 g (±1 g, Standard Error). The root system could be seen clearly at the base of the soil core.

2.3.1 Glomalin concentration and its spatial distribution

Fig. 2.3a & 2.3b show the spatial distribution of the glomalin concentrations in unplanted and planted treatments. No significant differences were detected between the planted and unplanted treatments ($P>0.05$). There were also no significant spatial differences in glomalin concentrations ($P>0.05$) in either planted or unplanted controls. The effect of the planted or unplanted condition upon the resulting spatial spread of data was also non-significant ($P>0.05$).

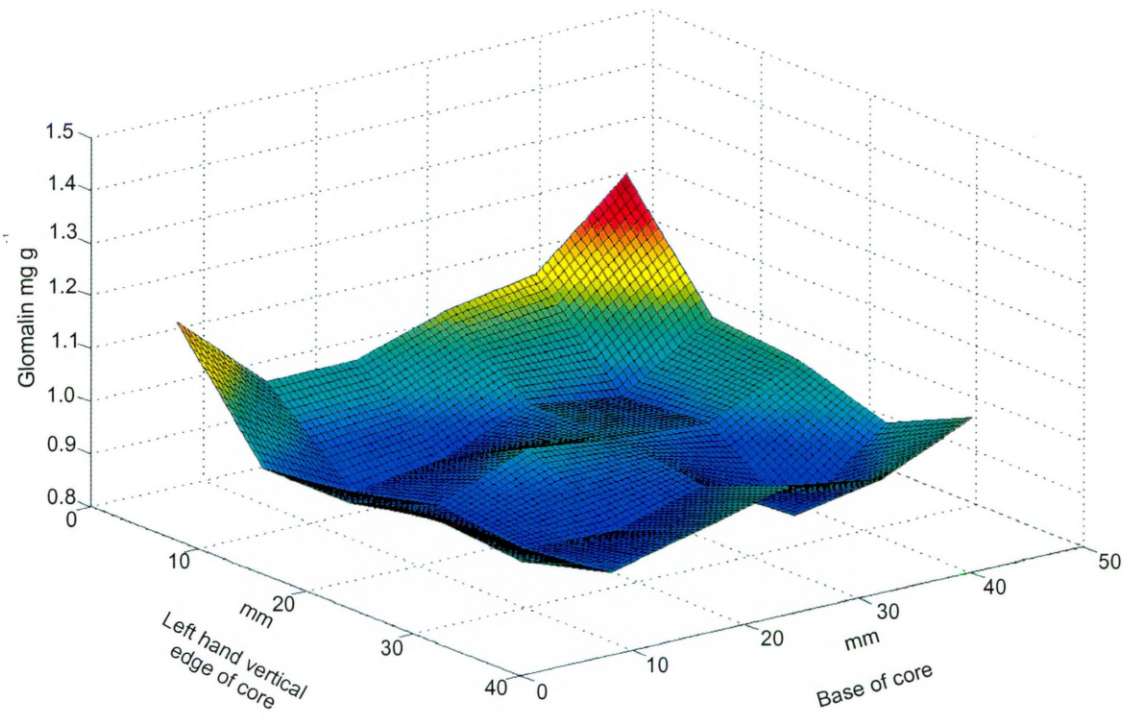


Fig. 2.3a Spatial plot of glomalin concentrations for unplanted cores packed with an arable soil. The data is not transformed.

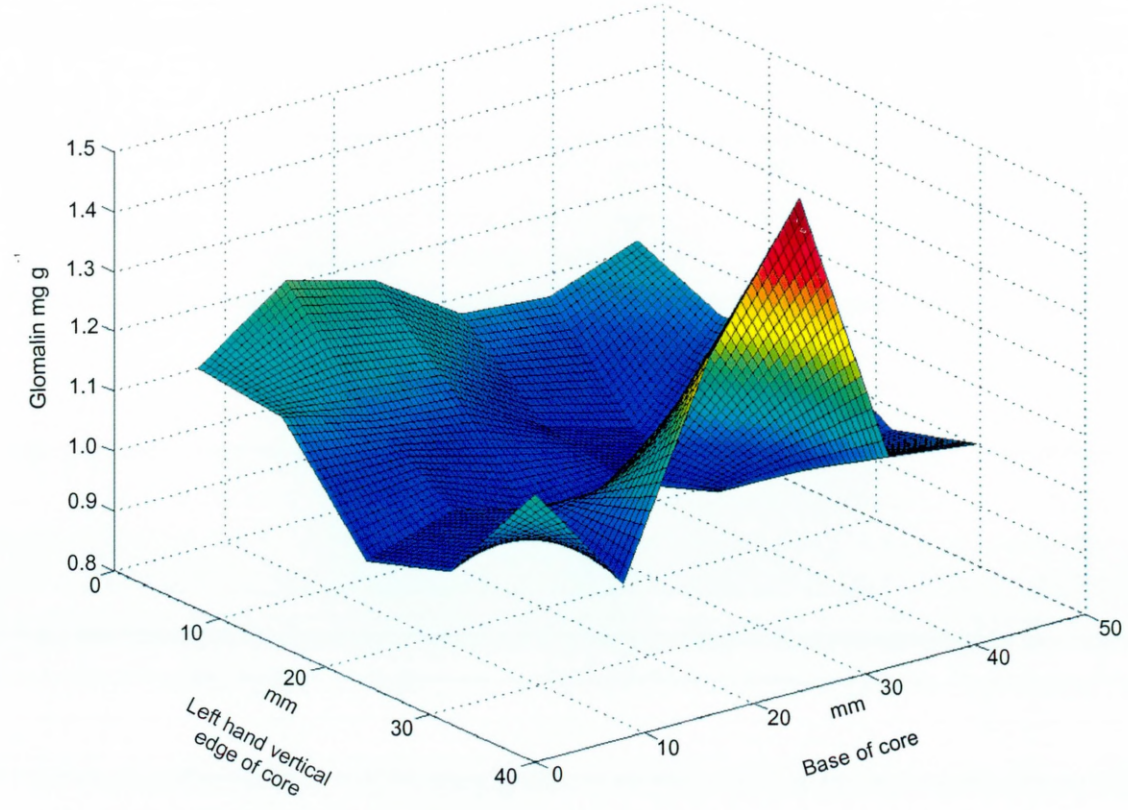


Fig. 2.3b Spatial plot of glomalin concentrations for planted cores packed with an arable soil. The data is not transformed.

2.3.2 Water repellency levels and its spatial distribution

Fig. 2.4a & 2.4b illustrate the spatial distribution of water repellency levels for both unplanted and planted treatments. A two-sample T-test applied to the Box Cox transformed water repellency data revealed significant differences between the planted and unplanted treatments ($P<0.01$), with the higher water repellency levels detected in the planted samples.

Water repellenecy levels did not show any significant spatial variation within core in either planted or unplanted cores ($P>0.05$). The spatial assessment did however, successfully detect differences in the spatial distribution of water repellency levels between planted and unplanted cores ($P<0.05$).

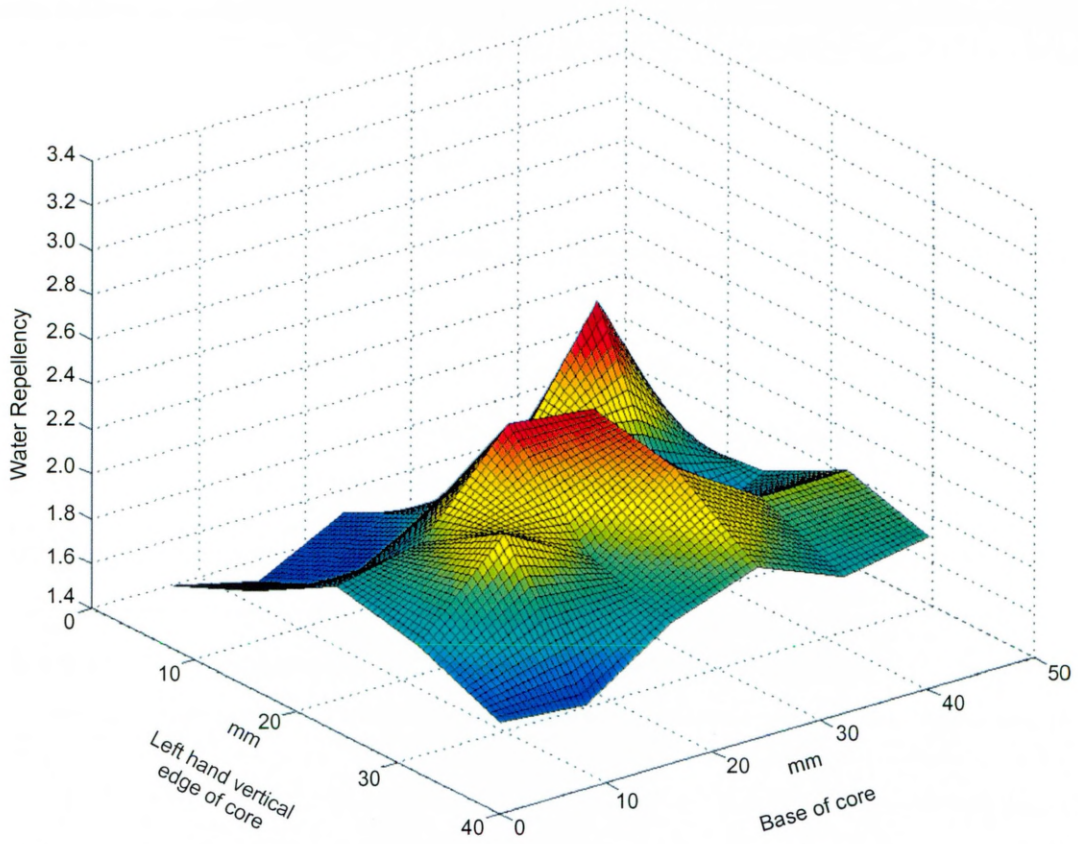


Fig. 2.4a Spatial plot of water repellency values for unplanted cores packed with an arable soil. The data is not transformed.

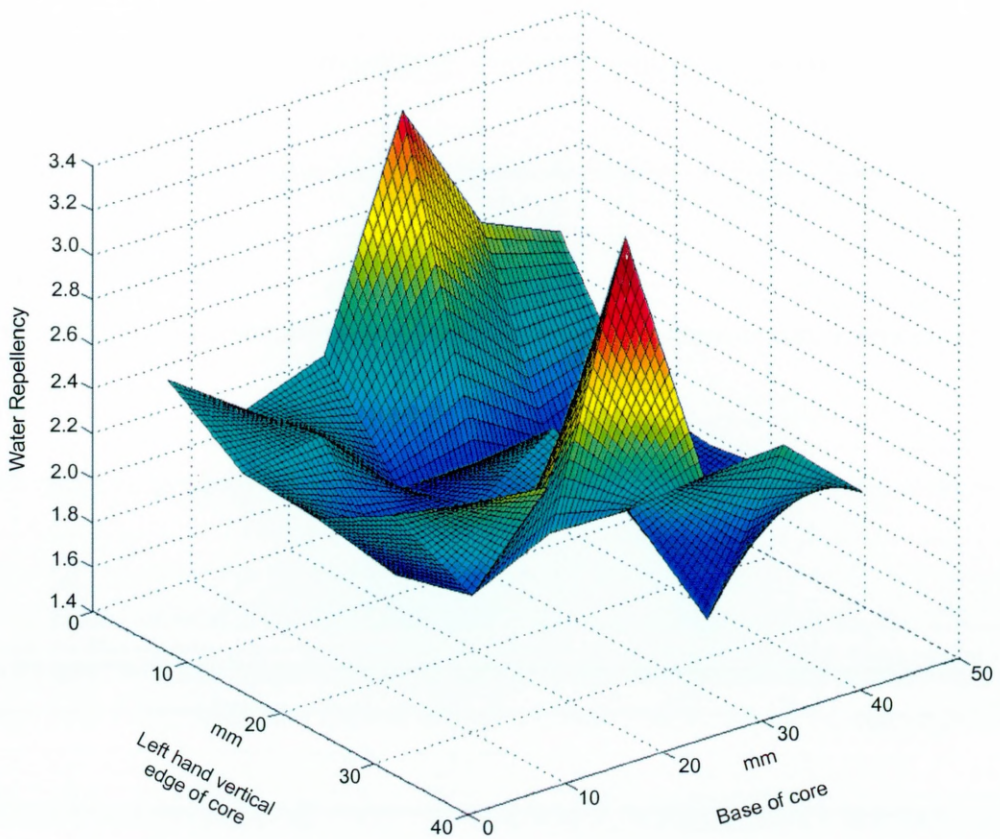


Fig. 2.4b Spatial plot of water repellency values for planted cores packed with an arable soil. The data is not transformed.

2.3.3 Correlation between glomalin and soil water repellency

Fig. 2.5 & 2.6 illustrates the relationship between glomalin and water repellency. A Spearman rank correlation was used to assess for any direct relationship between glomalin and water repellency. No direct relationship was detected under either unplanted r=0.15, or planted treatment r=0.21 (both P>0.05) between glomalin and water repellency.

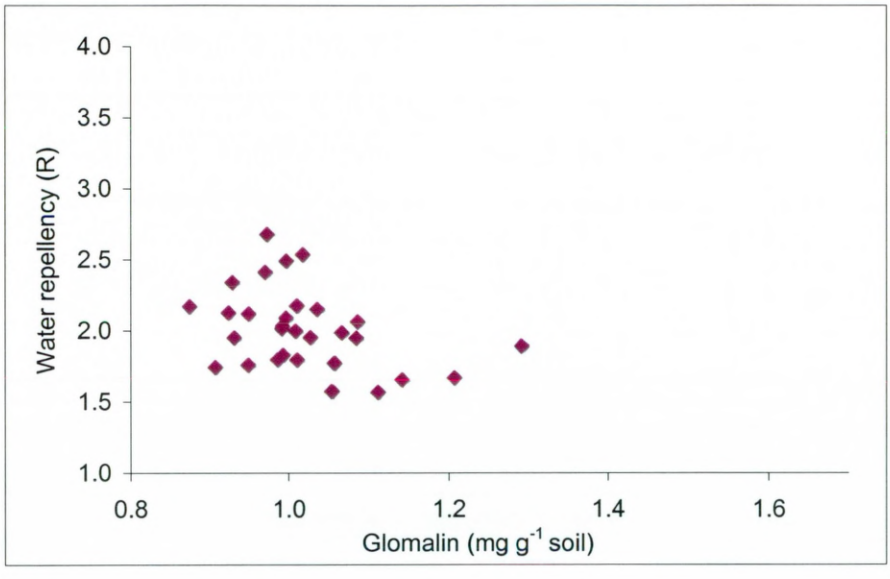


Fig. 2.5 Correlation of glomalin and water repellency values illustrated in a scatter plot for unplanted cores packed with an arable soil. Spearman rank correlation r=0.15, (P>0.05).

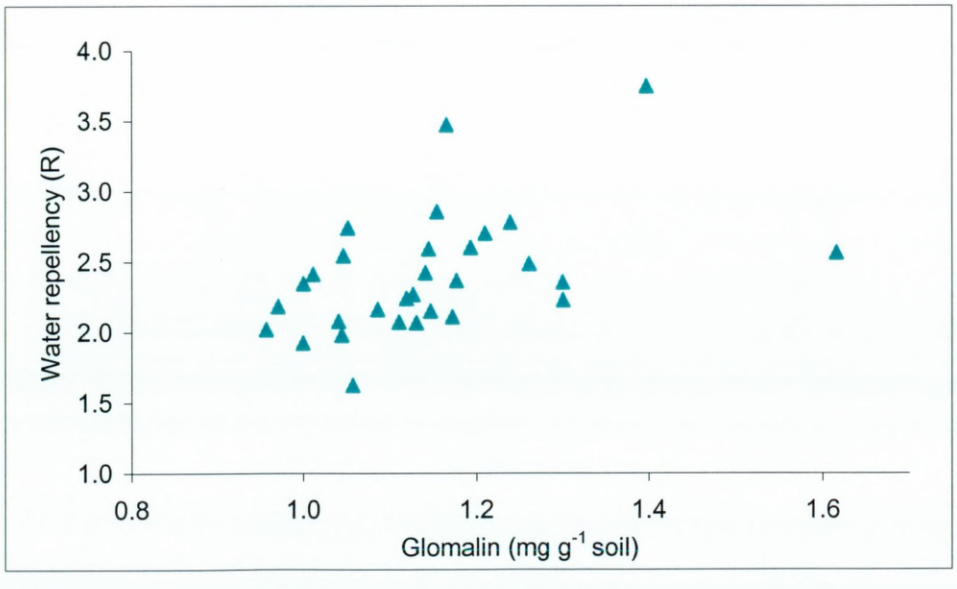


Fig. 2.6 Correlation of glomalin and water repellency values illustrated in a scatter plot for planted cores packed with an arable soil. Spearman rank correlation r=0.21, (P>0.05).

2.3.4 Mycorrhizal colonization of roots

The staining of root samples resulted in the positive identification of arbuscular mycorrhizal fungal structures. Spatially sampled roots using the grid sampling system showed varying levels of AM colonization with between 0-51% root lengths colonized (Table 2.1). Roots were present in 66% of soil blocks sampled, with 85% of root samples showing colonization by AM fungi. A sample root section colonized with AM hyphae is shown in Fig. 2.7.

Side of core	Top of Core					
	51.25		24.44	RP	8.93	8.47
			RP			35.44
		11.46	15.79		44.62	13.50
		22.96	37.70	30.00	34.73	29.50
			38.75	3.90	RP	10.72

Table. 2.1 Percentage root length colonization of root samples isolated from a spatially sampled soil core, samples with no visible root fragments present remain blank, whilst samples with roots present but no mycorrhizal colonization are represented as RP. Each cell represents a 1 cm soil cube sample.

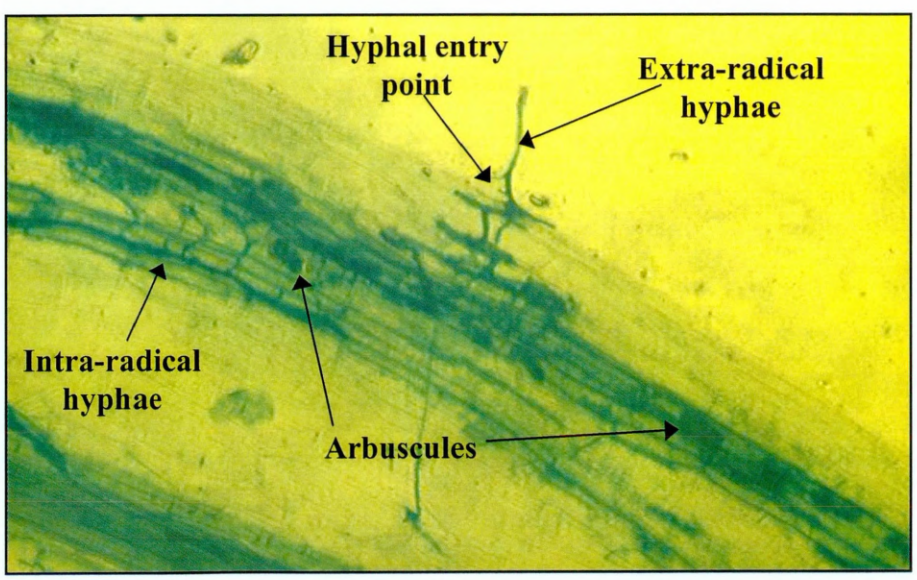


Fig. 2.7 Stained pea root section clearly colonized by mycorrhizal fungi viewed at 100× magnifications. Hyphae and hyphal structures are clearly visible with typical features marked.

2.4 Discussion

The hypothesis proposed by Wright and Upadhyaya (1998) that glomalin stabilises soil by reducing water infiltration was not validated in this study. Glomalin concentrations did not change significantly between the planted and unplanted treatments. Repellency, however increased significantly in planted cores suggesting that there is a biological impact on this property.

The lack of relationship between glomalin and water repellency could indicate that in this case glomalin did not impart water repellency. However, the lack of a significant increase in glomalin concentrations may be indicative that the incubatory period was too short, and that insufficient AM populations were established to detect any causal link between glomalin and water infiltration. Olsson and Wilhelmsson (2000) assessed the growth of AM fungi in sand dunes, and found no significant increase in hyphal growth between 35 and 68 days, suggesting that the maximal biomass of the AM population had been reached by 35 days. They also reported that the AM fungi grew at a rate of 1.2 mm per day. Hodge *et al.* (2001) also demonstrated large and significant growth of AM hyphae between 28 and 42 days. Therefore, the experimental set-up should have permitted sufficient hyphal proliferation throughout the soil, allowing a significant opportunity for glomalin to influence changes in soil properties.

No AM fungal inoculum was added to the soil with AM fungal colonization reliant of the *in situ* soil inoculm however, root sampling and staining positively identified the colonization of pea roots by AM fungi, and it is assumed that this would be the case for all planted soil cores. The understanding of the expression of this protein is not well understood. It could be possible that too much organic matter was removed from the soil restricting the amounts of protein expressed, or the sieving and storage of the soil in some way hindered the production of the protein. Further research is required to assess these factors.

Glomalin has been reported to be a resilient soil protein (Wright, 1997) and have almost recalcitrant properties, with Rillig *et al.* (2001) reporting a possible lifespan in the region of 6-42 years in soil. This may explain the similar levels of glomalin between the unplanted and planted treatments, particularly if the levels of glomalin exuded were insignificant. The relatively low concentrations of glomalin may not have been significant enough to directly impart water repellency. Without a considerable range of glomalin concentrations (in this case 1.0-1.2 mg g^{-1}) a comprehensive analysis of this relationship is difficult.

Significant increases in water repellency levels developed under planted conditions, despite no differences in glomalin concentration. Therefore, factors under rooted conditions significantly influenced water repellency, and thus, under these conditions these factors must be responsible for manifesting changes in soil water relations. The presence of the plant could have induced increased microbial activity, by increasing the input of carbon into the soil (Golchin *et al.*, 1994; Jastrow *et al.*, 1996). This hypothesised increase in microbial activity could well have been both bacterial and fungal (including AM fungi), which are known to influence soil water repellency (Bond and Harris, 1964; Miller and Wilkinson, 1977; Savage *et al.*, 1969; White *et al.*, 2000; York and Canaway, 2000) but in this case not through the proposed mechanism of glomalin exudation. Other authors have found strong links between microbial activity and water relations, either through pore blockage mechanisms under high substrate loads (Baveye *et al.*, 1998), or though increasing water repellency (Jex *et al.*, 1985; Savage *et al.*, 1972). Plant root mucilages have also been well documented to increase aggregate stability (Watt *et al.*, 1994), with Czarnes *et al.* (2000) reporting increases in soil water repellency after amendment of soil with synthetic root mucilage. Additionally, the localised drying of soil by roots (Allison, 1968) may further enhance the influence of plant root mucilages.

The spatial distribution of repellency values was different between planted and unplanted cores. Dexter (1988) described soil structures as "*the spatial heterogeneity of the different components or properties of soil*", and repellency may be a mechanism that enhances physical heterogeneity in soil. The planted cores had significantly greater repellency levels and a different spatial distribution, in contrast to unplanted cores, where it is hypothesised that microbial activity would be reduced.

Spatial heterogeneity in repellency was reported by Hallett *et al.* (Accepted 2004), who speculated that the influence of hydrophobic residues exuded by microbial populations and plants would be localised, and subjected to temporal variations. This was at a scale 20× larger than the current study and no independent analysis of biological properties was made.

The novel advancement of this work was the application of a spatial investigation and analysis of the relationship between glomalin and water infiltration, which demonstrated biologically induced spatial changes in repellency. The clear influence of plants upon repellency was either directly from roots or indirectly from microbial exudates, perhaps including AM fungi. The experimental conditions did not induce significant differences in glomalin concentration, however, as the soil cores were not inoculated with AM fungi it is possible that soil cores may not have developed a significant fungal population. The experimental set-up also consisted of the analysis of only one fixed time point, therefore it is unknown how quickly biologically induced repellency develops. To progress further from these results, the effect of roots and AM fungal hyphae need to be isolated to allow an accurate assessment of the impact of fungal hyphae (both AM and saprophytic) and their exudates upon soil water infiltration. The use of a further biomarker to indicate total fungal biomass, rather than just an AM fungal biomarker, would give an indication of how the increased presence of fungal hyphae affects soil water repellency.

2.5 Conclusions

The experimental set-up which assessed the levels of water repellency and glomalin in an arable soil incubated with and without pea plants resulted in the detection of increased water repellency in planted samples. However, changes in water repellency could not be attributed to the production of glomalin by AM-fungi, which has been hypothesised by other researchers as the major mechanism involved in soil stabilisation.

Clearly there are other biotic factors involved in repellency that need to be investigated. Root exudates may be repellent, or they may provide a substrate source for other organisms that create hydrophobic secondary metabolites or cellular structures. Without separating the effects of roots and fungi, it was not possible to determine if the resulting differences in water repellency were root or fungal induced.

Chapter 3: An investigation of the spatio-temporal relationships between fungi and soil water infiltration, as related to aggregation.

3.1 Introduction

Tisdall and Oades (1982) proposed the concept of aggregate hierarchy in soils, whereby transient and temporary agents bind soil particles into microaggregates (<250 μm) that in turn are bound into macroaggregates (>250 μm). The predominant factors of this conceptual model, backed by other researchers (Jastrow and Miller, 1998; Jastrow *et al.*, 1998; Miller and Jastrow, 1990, 1992, 2000; Oades, 1993; Oades and Waters, 1991; Six *et al.*, 1998) are that fungal hyphae and roots act as temporary agents physically binding and enmeshing soil particles to varying degrees (Jastrow and Miller, 1991; Tisdall and Oades, 1982). Aggregation can also be caused by bacteria and extra-cellular polysaccharides (exuded from hyphae, roots and bacteria), which bind particles like sticky glue, are transient agents (Miller and Jastrow, 2000; Wright and Upadhyaya, 1996; Tisdall and Oades, 1982).

Many researchers investigate changing environmental conditions upon the resulting soil aggregate size distribution or stability as a measure of soil health and stability (See Section 1.3.3). Typically, aggregate sizes researched are; microaggregates <53 μm and 53-250 μm, and macroaggregates 250-2000 μm and >2000 μm. Bossuyt *et al.* (2001) found that suppressing bacterial populations had little impact upon the formation of macroaggregates. They reported that the most significant impact upon formation of stable macroaggregates was that of fungal activity.

Whilst previous research has investigated the impact of soil fungi upon aggregate formation and stabilisation, the impact of AM fungi is often neglected, despite research showing their importance in binding and enmeshing effects along with roots (Miller and Jastrow, 1990, 1992; Tisdall and Oades, 1982; Tisdall *et al.*, 1997). AM hyphae can grow approximately 8.5 cm from the root (Olsson and Wilhelmsson, 2000) and there is very little understanding of this possible zone of influence. Thomas *et al.* (1993) separated the effects of roots and AM fungi, investigating root length and hyphal density, and compared these with the amount of water stable aggregates (WSA). They found changes in the quantity of WSA related to the presence of AM fungi, thereby proposing that the hypothesised impact was as a result of hyphae slowing the slaking process. However, no comparable (non-

incubated) initial measure was made and thus comparisons were only between experimental conditions and an internal control. Reduction in slaking is also the hypothesised mode of action proposed for glomalin induced changes in aggregate stabilisation (Wright and Anderson, 2000; Wright *et al.*, 1999, Wright and Upadhyaya, 1998) (and also Section 1.4.2), a factor that was investigated in Chapter 2.

Studying a number of agricultural soils Guggenberger *et al.* (1999a) found that within macroaggregates (>250 μm) the microbial community was dominated by fungi. Guggenberger *et al.* (1999a) found that the physical entanglement of microaggregates persisted even after cell death of fungi, indicating that another factor may play a significant role in soil stabilisation. The other factor that Guggenberger *et al.* (1999b) proposed in addition to physical entanglement was changes to the wetting behaviour of test soils. They observed "*pronounced water repellency*" of soil surfaces when wet sieving, proposing that the impact of hydrophobic compounds had a significant effect upon soil stability. Caron *et al.* (1996) also reported that differences in levels of wetting were related to soil stability, and reported that soils displayed increased wetting rates alongside decreases in stability. The observations by previous researchers hypothesise a relationship between wetting rates (or subcritical repellency) and soil aggregate stability, but to date have failed to measure water repellency along with aggregate stability in order to substantiate this link. Further to this is the proposed influence of glomalin upon aggregate stabilisation, which is proposed to impede soil wetting (Wright and Upadhyaya, 1998) and increase soil stability. However, much of this work has been completed on structurally stable soils, therefore a comparison of a structurally stable and unstable soil would be a logical further investigation.

The aim of this chapter is to examine both separately and in combination the effect of fungi and roots, examining the formation of micro-aggregates and macro-aggregates using a time course study. Included in the investigation are a structurally unstable soil prone to slaking and a stable non-slaking soil. Griffiths and Young (1994) reported that Lab Field soil was structurally unstable when immersed in water and slaked immediately, whilst Bullion Field soil is reported to be structurally stable (Preston, 1997 unpublished PhD thesis). The investigation of stabilisation

mechanisms using a slaking and non-slaking soil will allow an appropriate comparison of the mechanistic assessment of the impact of roots, fungal hyphae and glomalin.

Soils were seeded with *Lolium perenne,* and roots were contained within a nylon mesh, which allowed fungal hyphae to penetrate the soil outwith this area, in order to assess the effect of fungi (both AM and saprophytic) upon soil properties. The impact of fungal biomass (through measures of ergosterol) and the AM fungal protein glomalin upon the wetting properties of soil were assessed and the subsequent effects of these changes were used to detect for a hypothesised positive relationship with aggregate size distribution.

3.2 Materials and methods

3.2.1 Soil core preparation

Both soils were situated at the Scottish Crop Research Institute and were selected for differences in structural stability. Bullion Field is a structurally stable soil, whilst Lab Field is a structurally poor soil that is prone to slaking (Griffiths and Young, 1994). Details of the soil characteristics are detailed in Table 3.1.

Soil	Sand %	Silt %	Clay %	% C	%N	C:N	pH
Bullion Field	71	19	10	2.99	0.22	13.6	6.2
Lab Field	59	34	7	1.9	0.07	27.1	5.94

Table 3.1 Soil characteristics of Bullion Field and Lab Field soils.

Both soils were sampled from the top 100 mm of fallow plots and subsequently sieved at field water content to 2 mm.

The soils were packed into cores (diameter of 103 mm and a height of 55 mm). The initial gravimetric water content was measured (Bullion Field: 14% ±0.08, Lab Field: 17% ±0.05) and the soils were brought to a water content of 20%, which is

close to field capacity. Soils were then packed to a dry bulk density of 1.3 Mg m^{-3}. For each soil a total of 40 unplanted control cores were prepared. An additional forty planted cores were prepared for each soil that had an isolated central region 55 mm in height and 60 mm in diameter. The separate central region was created by packing the entire core to the desired bulk density and then inserting a second smaller core into the centre of the larger core. The central region was removed, covered in a 38 μm nylon mesh to contain roots but not fungal hyphae, and then placed back into the original core. The central rooted region will be referred to as planted inner, the un-rooted external section will be referred to as planted outer, and the control cores will be referred to as unplanted.

The arbuscular mycorrhizal fungal inoculum of *Glomus mosseae* was taken from a pure pot culture maintained on *Plantago lanceolata* on 50:50, sand:Terragreen mix (OIL-DRI, UK). Pot cultures were maintained in growth cabinets at 15°C, 60% humidity with a 16 h day 8 h night cycle. Cultures were tested for root colonization and sub-cultured after an incubatory period of three months by sampling root and hyphal material from colonized pots and seeding fresh pots containing washed and sterilised sand/Terragreen mixture. Throughout the growth period plants and fungi were supplemented with Rorison's nutrient solution, which, supplies magnesium, calcium, potassium and other trace element nutrients and contained low concentrations of phosphate (University of York: Mycorrhiza Research Group, 1999). Spores were isolated from dried pot cultures by wet sieving (710 μm sieve), and subsequent centrifugation (1800 rpm for five minutes). After centrifugation the spores were added to a sucrose solution (440 g L^{-1}) and centrifuged for a further 5 minutes (1800 rpm) spores were isolated from the resulting supernatant by sieving through a 32 μm sieve (viable spores floated in sucrose solution), spores were washed thoroughly with water to remove osmotic stress. This process ensured the removal of viable spores without any additional organic material. Approximately 150 (±21) spores were added to the top 20 mm of the central region of the cores and covered in soil. *Lolium perenne* was chosen as a host plant, with an average of 117 (±4) seeds added to the planted inner region by sprinkling evenly across the surface of the core, and then covered with a thin (5 mm) layer of soil.

All cores were covered with a thin layer of black PVC beads to reduce evaporation. The soil cores were incubated in a growth chamber at 15°C, 80% humidity with a 16 h day 8 h night cycle (Fitotron: 40, 000 LUX, 500 mmol $m^{-2}s^{-1}$, 110 W m^{-2}). Cores were watered to their initial weight every 1 to 2 days to keep them at a mass of 20% water. Fig. 3.1 illustrates the experimental set-up.

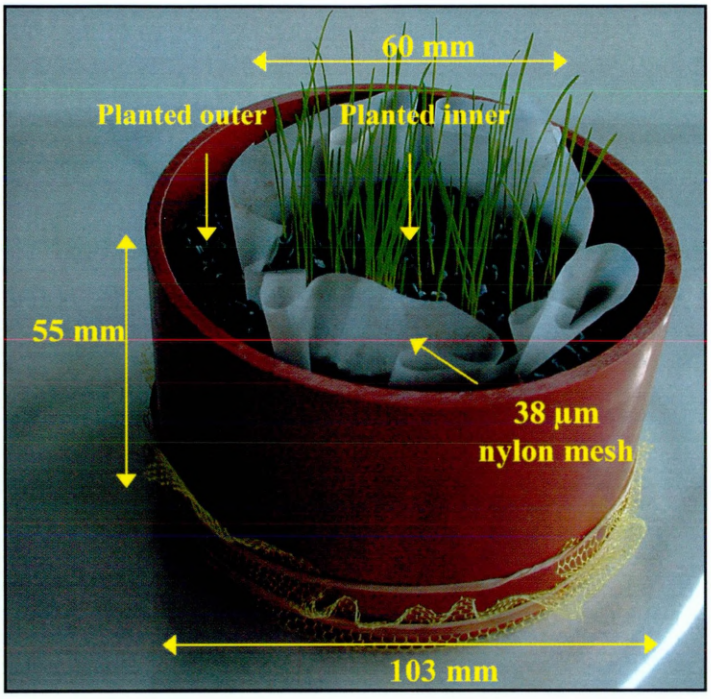


Fig. 3.1 Split soil core system, highlighting the "planted inner" and "planted outer" divisions. Separate plant-free cores were prepared for the unplanted treatment.

3.2.2 Core sampling

Cores were sampled after 0, 7, 9, 11, 13, 15, 17, 20 and 30 days incubation, with five replicate cores per sampling day. The core weights and the lengths of roots and shoots were recorded at sampling. Sampling was destructive; cores were taken apart with soil sampled from unplanted treatments and both planted inner and planted outer sections of cores. Replicate soil samples were selected throughout the soil cores for extracted ergosterol and glomalin analysis (Sections 3.2.3 & 3.2.4). Soil to

be assessed for water infiltration and aggregate size distribution was placed into aluminium trays and dried at 40°C overnight.

3.2.3 Assessing the sensitivity of ergosterol isolation procedures

Ergosterol isolation was based on the extraction procedure of Ruzicka *et al.* (1995). In order to reduce the volume of solvent and soil used in the extraction process, and to increase the sample throughput, three ergosterol extraction procedures were tested. Four different soil samples were tested: three samples from Bullion Field under various vegetation types, bramble, fallow, grass and hedge and one fallow plot from Lab Field (further soils details in Section 3.2.1). This provided a range of vegetative cover and soils.

For all treatments 0.3 g of soil was weighed into a micro-centrifuge tube along with 400 µl of a Methanol:Ethanol mix (4:1). All soils were incubated at <4°C for 2h. A further 1.2 ml of Isohexane:Propan-2-ol (98:2) was added and then one of the following three disruption methods was used:

1. **Bead-beating**: 0.1 g of 1 mm glass beads was added to samples, which were bead-beaten on a medium speed setting (MiniBeadbeater-8, Biospec Products) for one minute.

2. **Vortexing**: Samples were vortexed (Whirlimixer, Fisons), maximum speed setting for one minute.

3. **Sonication**: Extracts were sonicated for 250 seconds using a Microscon XL2005 sonicator (level 5); samples were maintained over ice during sonication to prevent degradation of ergosterol due to heating.

After disruption, samples were placed on ice and allowed to settle. The top 1 ml of the liquid phase was removed and centrifuged at 15,700 g for 10 minutes. The top 900 µl of the supernatant was syringe filtered through a Whatman 0.2 µm (4 mm) filter. The resulting solution was subjected to reverse phase high performance liquid chromatography (HPLC) analysis using a Waters 1525 Binary HPLC pump set at

282 nm. The column was a stainless steel cartridge (15 mm × 46 mm) packed with Li Chrosorb S160-5. The mobile phase was Isohexane:Propan-2-ol (98:2) with a flow rate of 1.5 ml min^{-1}; 25 µl of sample was injected and the retention time of ergosterol under these conditions was between 6.4 to 7.1 minutes.

3.2.3.1 Preliminary ergosterol isolation results

Fig. 3.2 shows the results of the various extraction procedures. For all soils, bead-beating consistently extracted the greatest concentration of ergosterol ($P<0.01$). Bead-beating also had the smallest coefficient of variation in comparison with the other methods, indicating it was a more consistent isolation procedure. The scaled down volume of solvents used, and changes made to the disruption procedure, allowed an increase in the throughput of samples for extraction, whilst maximising ergosterol isolation.

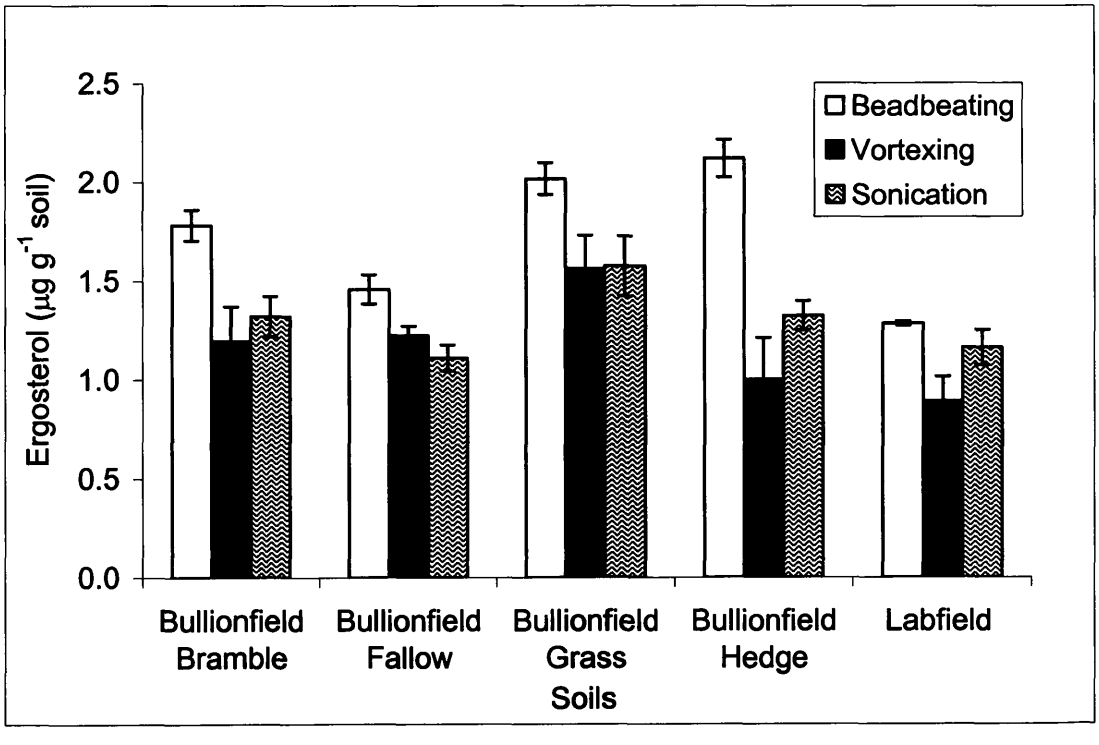


Fig. 3.2 Resulting ergosterol concentrations for the three extraction procedures, chart show averages of five replicate extractions (errors bars are standard error of the mean).

As a result of the preliminary extraction analysis, beadbeating was selected as the extraction procedure. Three replicate ergosterol measurements were made for unplanted, planted outer and planted inner treatments within each core for every time point.

3.2.4 Glomalin

Three replicate glomalin measurements were made for unplanted, planted outer and planted inner treatments within each core for each time point. Glomalin was extracted as detailed in Section 2.2.3.

3.2.5 Water repellence

For each of the treatments (i.e. unplanted, planted outer and planted inner), three replicate water repellency measurements were made per core. Measurements were taken at randomised regions within the centre of soil cores. Water repellency was measured as detailed in Section 2.2.4.

3.2.6 Root staining

Roots were initially washed in deionised water to remove attached soil and stained with 0.05% trypan blue (Phillips and Hayman, 1970). The percentage root length colonization was quantified using the grid-line intersect method (Giovannetti and Mosse, 1980). A full description of both staining and quantification is detailed in Section 2.2.5.

3.2.7 Aggregate size distribution

Aggregate size distribution was measured using a method described by Elliott (1986) and adapted by Six *et al.* (2000). Briefly, approximately 50 g of dried (overnight at 40°C) soil per sample was wet sieved through three different sieves in order to detect differences in the size distribution for each treatment. Soil was slaked immediately prior to disruption by placing on to the 2000 μm sieve filled with deionised water

and leaving for 5 minutes before any disturbance was applied. To disrupt soil, the sieve was moved up and down for two minutes. Material remaining on the sieve was washed into a container and dried at 40°C. Material that passed through the sieve was washed over 250 μm and then 53 μm sieves in the same manner as described previously. The resulting dried fractions were weighed and calculated as percentages of the original sample. One measure of aggregate size distribution was made per core with five replicates cores per time point.

3.2.8 Microaggregate size distribution

Three time-points from the Lab Field soil core experiment were subjected to microaggregate size distribution analysis, in the range of 1-100 μm. Lab Field soil was selected for this analysis as it was considered to be structurally poorer (than Bullion Field soil), thus changes at the microaggregate scale as a result of incubation would be more likely to be detected in this soil. The samples were selected to give an indication of changing microaggregate size for the whole experimental period, replicate samples for zero day, 15 day (unplanted, outer planted and inner plated) and 30 days incubation (unplanted, outer planted and inner planted) were selected. Five replicates of each sample were analysed. Initially samples were air-dried and sieved to less than 109 μm, with 5-6 g of the sieved soil mixed with 100 ml of deionised water and shaken by hand for thirty seconds and then poured into the mixing chamber of a SediGraph 5100 (Micrometrics Instrument Corporation, Norcross, GA.). The mixing chamber was used to continually homogenise the sample suspension. After a fixed time the disturbance ceased and the measurement of falling particles/microaggregates began. Using an X-ray beam the SediGraph measures microaggregate size distribution through the falling or settling rate of particles within a known depth, time and area. Stokes' Law is used to establish particle size and this describes the way in which different sized particles fall in a volume of liquid, with larger particles falling more rapidly than smaller particles. The Sedigraph was set for the detection of particles/microaggregates between 109 and 1.8 μm.

The output of the Sedigraph gives the cumulative mass fraction for each microaggregate size. This value was recalculated into the proportion (of total sample) and analysis was completed on these values. The breakdown of aggregate sizes for analysis were the same as those selected by Watts *et al.* (2001): 100-50, 50-20, 20-10, 10-5, 5-2 and >2 μm.

3.2.9 Statistical analysis

An Anderson-Darling normality test showed that the data sets were highly skewed ($P<0.05$). Differences between treatments and time points were therefore assessed using a Kruskal-Wallis test, whilst relationships between measurements were tested for significance using a Spearman rank correlation.

3.3 Results

3.3.1 Temporal differences in fungal biomass

The effects of incubation upon fungal biomass expressed as ergosterol concentrations for Bullion Field and Lab Field soils, are shown in Fig. 3.3a &b respectively. Both soils showed significant increases in ergosterol concentration ($P\leq0.001$). For Bullion Field soil there was a similar trend in ergosterol concentration for the different treatments throughout the experiment. The concentrations between the treatments were similar until days 20 and 30 when the planted inner treatment showed significant increase in ergosterol concentrations ($P<0.05$).

Under Lab Field soils there were no significant differences between the treatments at any time point ($P>0.05$), with all treatments increasing to similar concentrations. Initial concentrations of ergosterol were 1.2 μg g^{-1} under unplanted day zero samples increasing to 2.7 μg g^{-1} under the planted inner section after thirty days incubation.

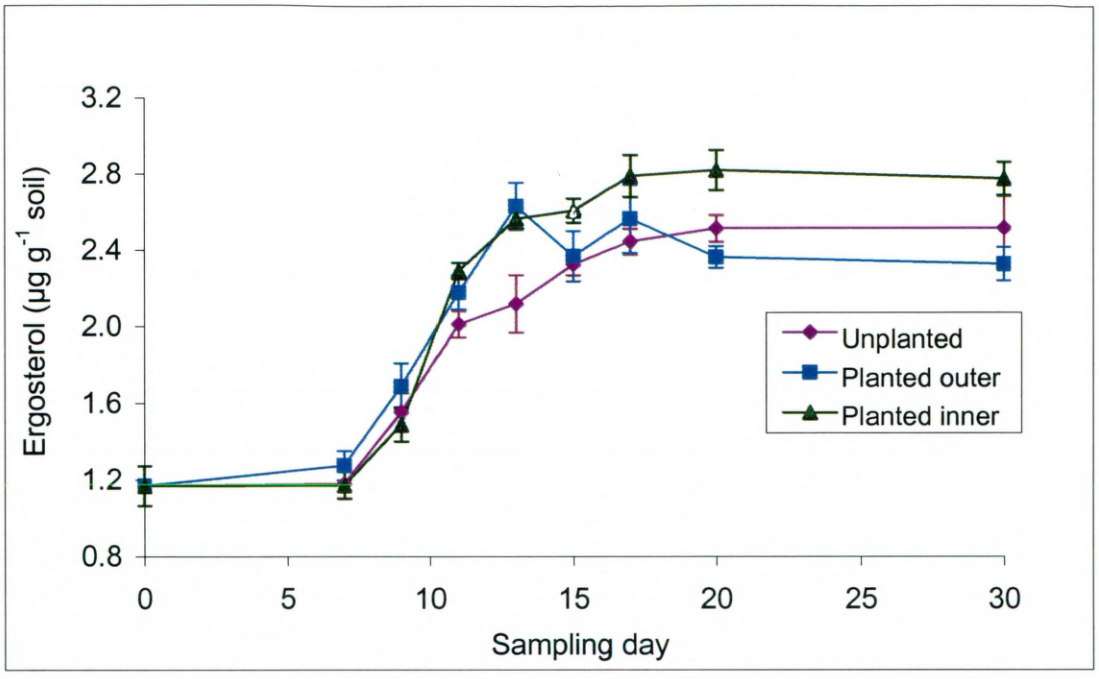


Fig. 3.3a Bullion Field soil, incubation and treatment effects upon ergosterol levels (error bars indicate standard error of the mean).

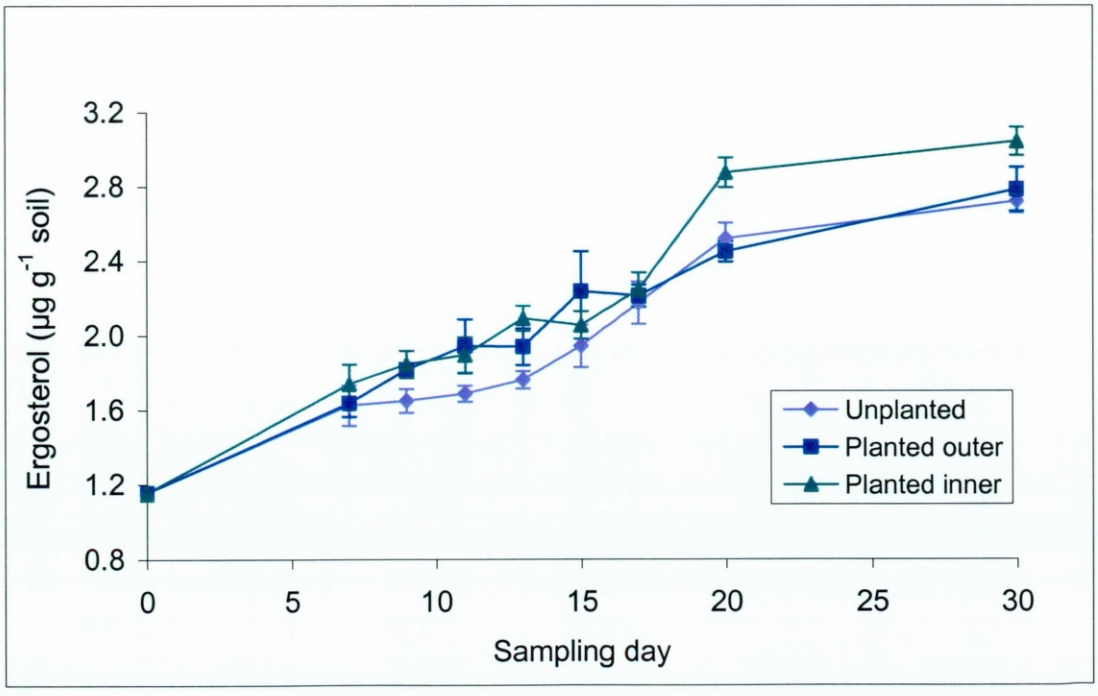


Fig. 3.3b Lab Field soil, incubation and treatment effects upon ergosterol levels (error bars indicate standard error of the mean).

3.3.2 Changing glomalin concentrations

The effects of incubation upon glomalin concentrations expressed for Bullion Field and Lab Field soils are shown in Fig. 3.4a &b respectively. The two soils differed considerably in glomalin concentration with greater concentrations of glomalin detected in Bullion Field soils. In Bullion Field soil the unplanted treatment showed no significant changes in glomalin concentration throughout the incubatory period ($P>0.05$). Within planted outer and inner sections, however, there were significant increases in glomalin concentration over time (both $P<0.01$). The planted outer treatment showed a steady and relatively unchanging concentration of glomalin until day 17, after which there was a significant increase ($P<0.01$) in concentration from day 20 through to 30. Glomalin increased more rapidly in the planted inner treatment, with a maximum concentration reached between days 15-20, rising from 1.4 mg g^{-1} soil on day zero to 2.2 mg g^{-1} on day 20 ($P<0.01$). Interestingly, there was a significant drop in concentration between days 20 and 30 ($P<0.01$).

Lab Field soil expressed no statistically significant differences, either throughout the incubatory period or between treatments ($P>0.05$). Higher glomalin concentrations were detected under the planted inner treatment compared to the unplanted and planted outer on days 13, 15 and 17 ($P<0.05$) but on the final sampling point, day 30, no significant differences between the treatments were detected ($P>0.05$).

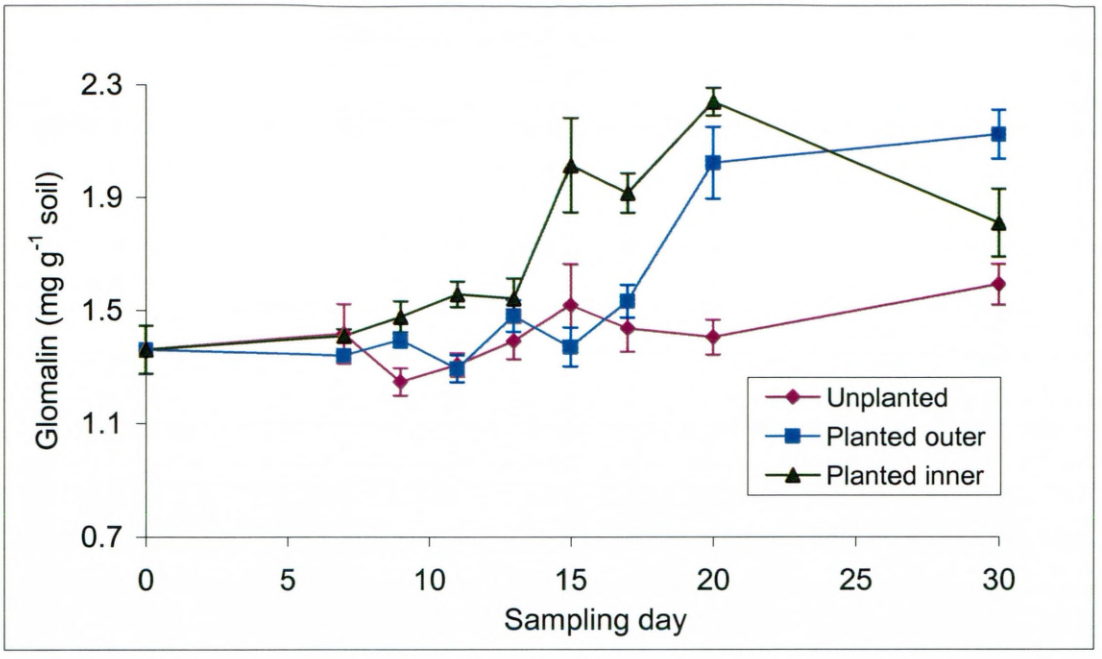


Fig. 3.4a Bullion Field soil, incubation and treatment effects upon glomalin levels, (error bars indicate standard error of the mean).

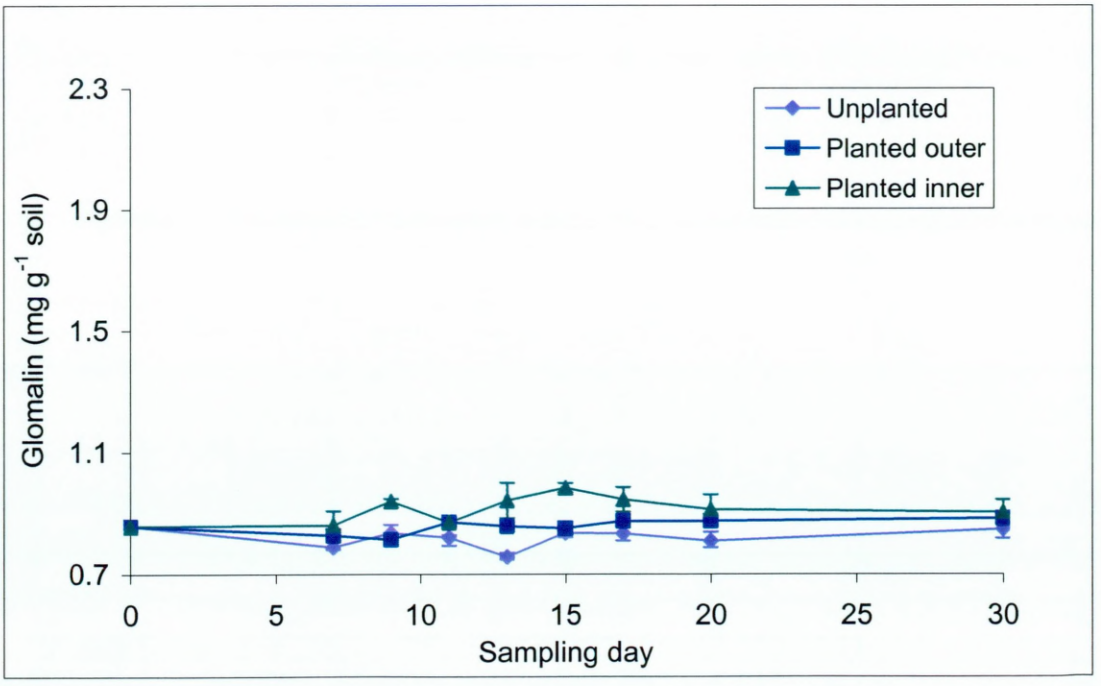


Fig. 3.4b Lab Field soil, incubation and treatment effects upon glomalin levels (error bars indicate standard error of the mean).

3.3.3 Root fungal colonization

In order to establish the effect of the fungal inoculum and the extent of fungal colonization throughout the experiment, the average percentage root length colonization was assessed for each time point. The resulting root length colonization for Bullion Field and Lab Field soils are shown in Fig. 3.5a &b respectively.

After only seven days incubation, the staining and observation process positively detected the presence of AM fungi colonizing root samples. Under both soils the percentage of root length colonized steadily increased over the time period.

Roots in Bullion Field soil reached a maximum length colonized between 17-20 days incubation, after this time the level of colonization showed a slight drop on day 30. Whilst under Lab Field soil the maximum average of 21% of the root length colonized was detected at the final day's sampling.

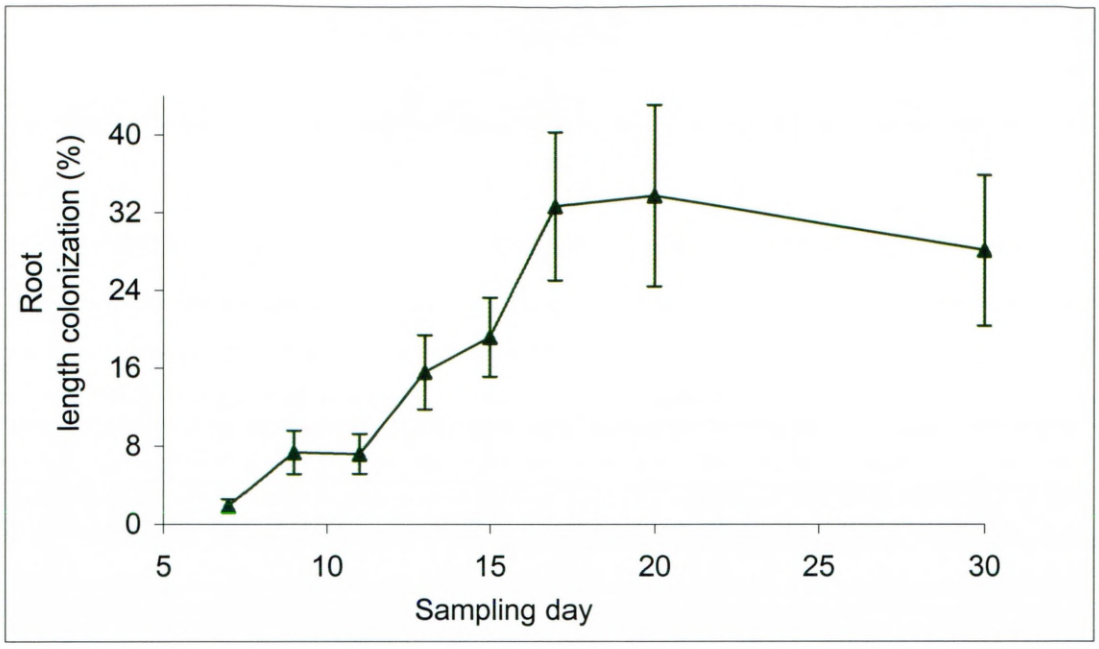


Fig. 3.5a Bullion Field soil, percentage root length colonization throughout incubatory period (error bars are standard error of the mean).

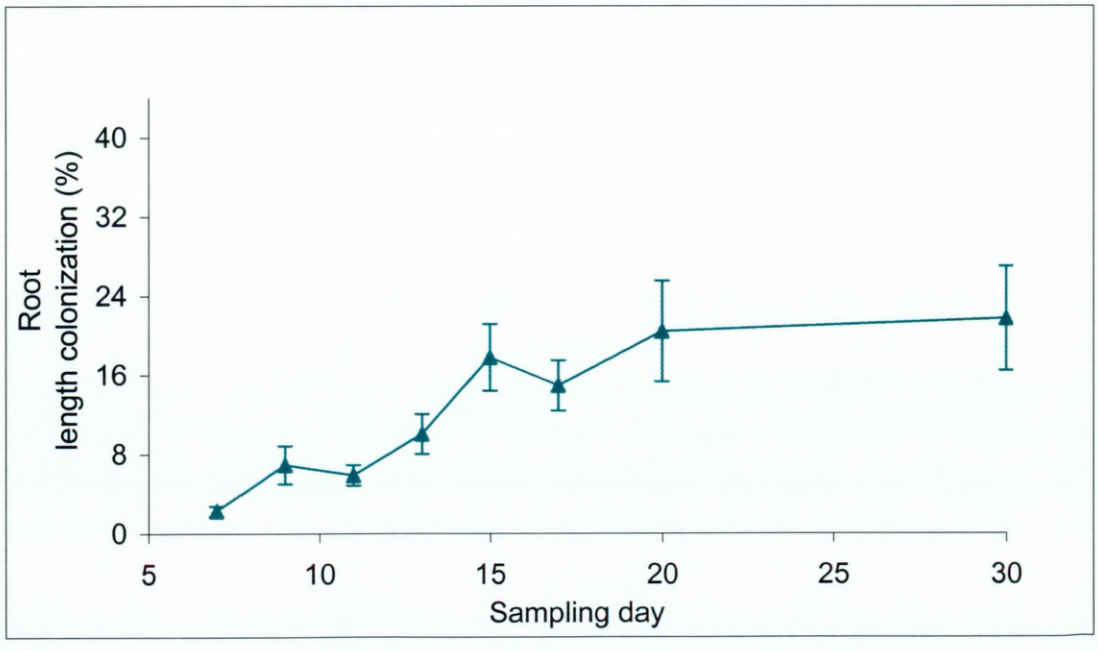


Fig. 3.5b Lab Field soil, temporal percentage root length colonization throughout incubatory period (error bars indicate standard error of the mean).

3.3.4 Changing levels of water infiltration

Levels of water repellency detected throughout the incubatory period for Bullion Field and Lab Field soils are shown in Fig. 3.6a & b respectively.

Under both soils significant changes in water repellency were detected throughout the experimental period under all treatments ($P<0.001$). In Bullion Field soils unplanted and planted outer samples both had similar trends in repellency levels, and no significant differences between the two treatments were detected at any time point ($P>0.05$). The planted inner levels of repellency reached a peak at sampling day 17 with a repellency value of 4.8, but then subsequently fell to 3.7 by sampling day 30, a value that was still significantly greater than the day zero when no repellency was detected. Under Bullion Field soil the planted inner treatment consistently expressed significantly greater levels of water repellency that the other two treatments at all time points ($P< 0.05$).

Under Lab Field soil repellency increased from 1.2 on day 0 to a maximum of 2.9 on day 30 (planted outer). On day thirty there were no significant differences detected between the three treatments ($P>0.05$).

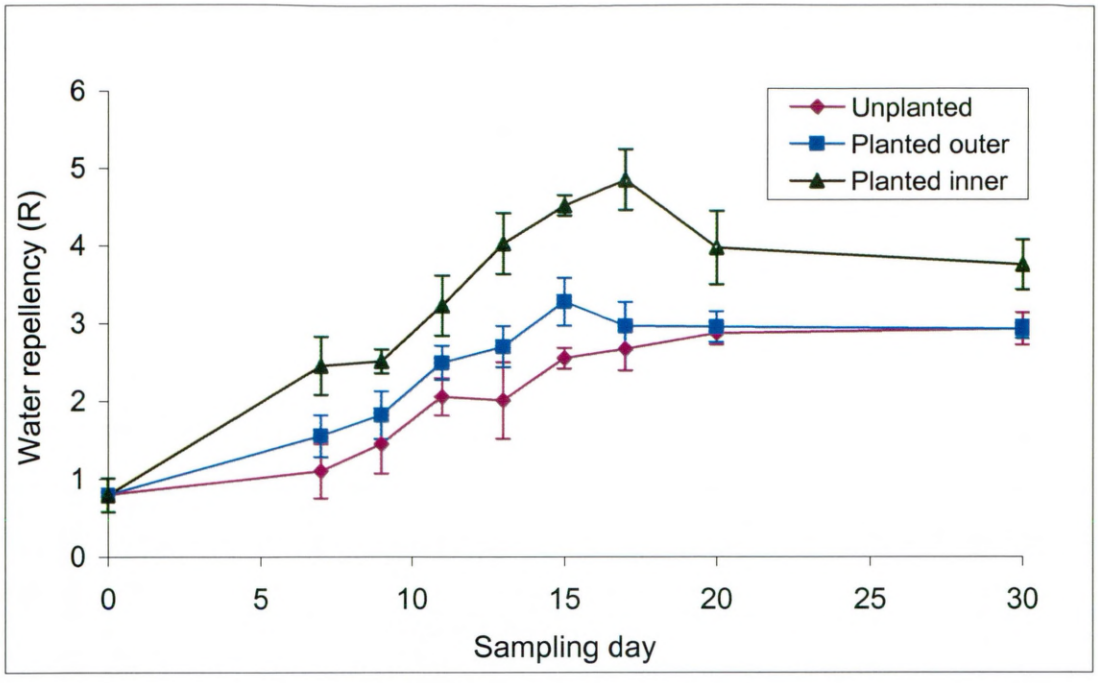


Fig. 3.6a Bullion Field soil, incubation and treatment effects upon water repellency levels (error bars indicate standard error of the mean).

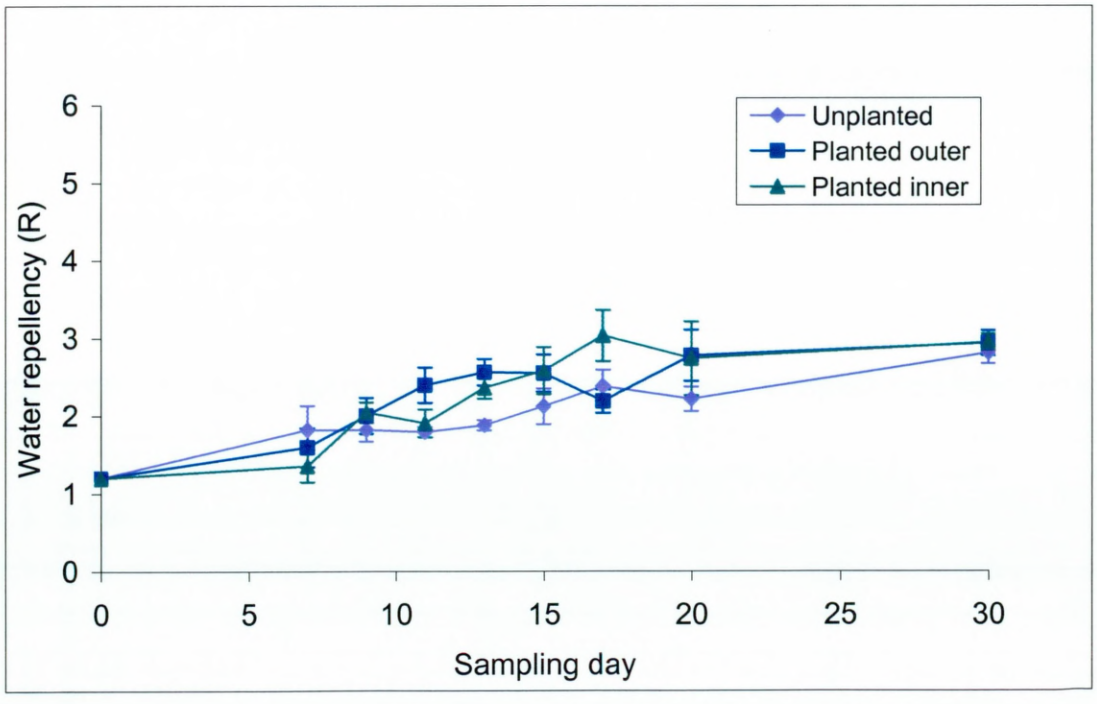


Fig. 3.6b Lab Field soil, incubation and treatment effects upon water repellency levels (error bars indicate standard error of the mean).

3.3.5 Aggregate size distribution

The resulting breakdown of aggregate size classes is expressed proportionally as the percentage of the original sample; these results are illustrated in Fig. 3.7 (Bullion Field: a, c, e & g; Lab Field: b, d, f & h). Prior to incubation the test soil was sieved to 2000 μm, hence on day zero there were no water stable aggregates >2000 μm. Therefore, subsequent increases in aggregates of this size could be attributed to experimental perturbations.

Both soils showed significant increases in the percentage of >2000 μm aggregates ($P \leq 0.001$) under all treatments, with Bullion Field having a greater proportion of aggregates in this size class in contrast to Lab Field soils. The largest increase in aggregates of this size class was detected under the planted inner treatment, which continued rising with total of 24.4% for Bullion Field and 17.2% for Lab Field recorded on day 30. To a lesser extent both unplanted and planted outer treatments showed considerable increases rising to 7.0% and 10.2%, respectively for Bullion Field and 6.2% and 8.0% for unplanted and planted outer, respectively for Lab Field.

Aggregates 250-2000 μm in Bullion Field showed no significant changes under the unplanted treatment ($P>0.05$). However, significant changes throughout the incubatory period were detected under planted outer and planted inner treatments ($P<0.05$) with both treatments showing an initial increase in 250-2000 μm aggregates then a subsequent decline. Lab Field soil, however, demonstrated no significant differences between treatments or time points ($P>0.05$) for proportions of aggregates in the size class 250-2000 μm.

Under Bullion Field soil no significant differences were detected in proportions of aggregates 53-250 μm either between treatments or throughout the incubatory period ($P>0.05$). Additionally fine particles or aggregates <53 μm showed a general reduction in all treatments, however, these differences were also not significant

either between treatments or time points ($P>0.05$). Under Lab Field soil the size classes 53-250 μm and <53 μm both showed a significant decline in proportion between 0 and 30 days incubation under all treatments ($P\leq0.001$ for both size classes). No differences between the treatments were detected in either 53-250 μm or <53 μm proportions ($P>0.05$).

The resulting proportions of aggregate sizes for each experimental soil are illustrated in Fig.3.7a-f.

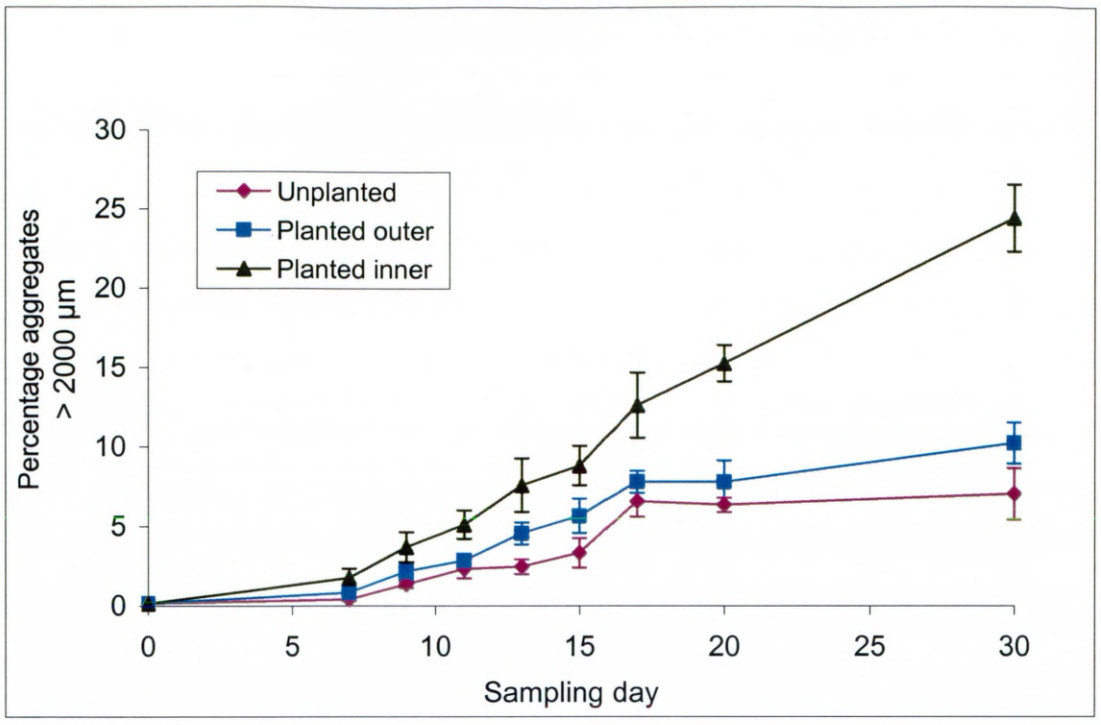


Fig. 3.7a Bullion Field soil, incubation and treatment effects upon percentage of aggregates >2000 µm (error bars indicate standard error of the mean).

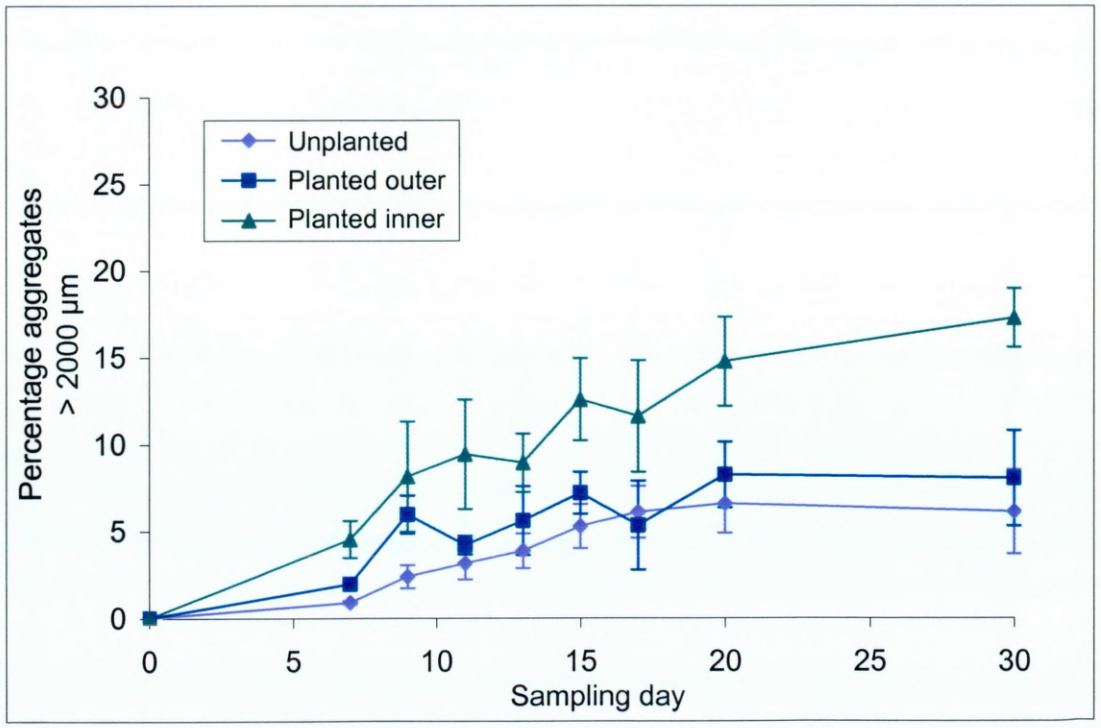


Fig. 3.7b Lab Field soil, incubation and treatment effects upon the percentage of aggregates >2000 µm (error bars indicate standard error of the mean).

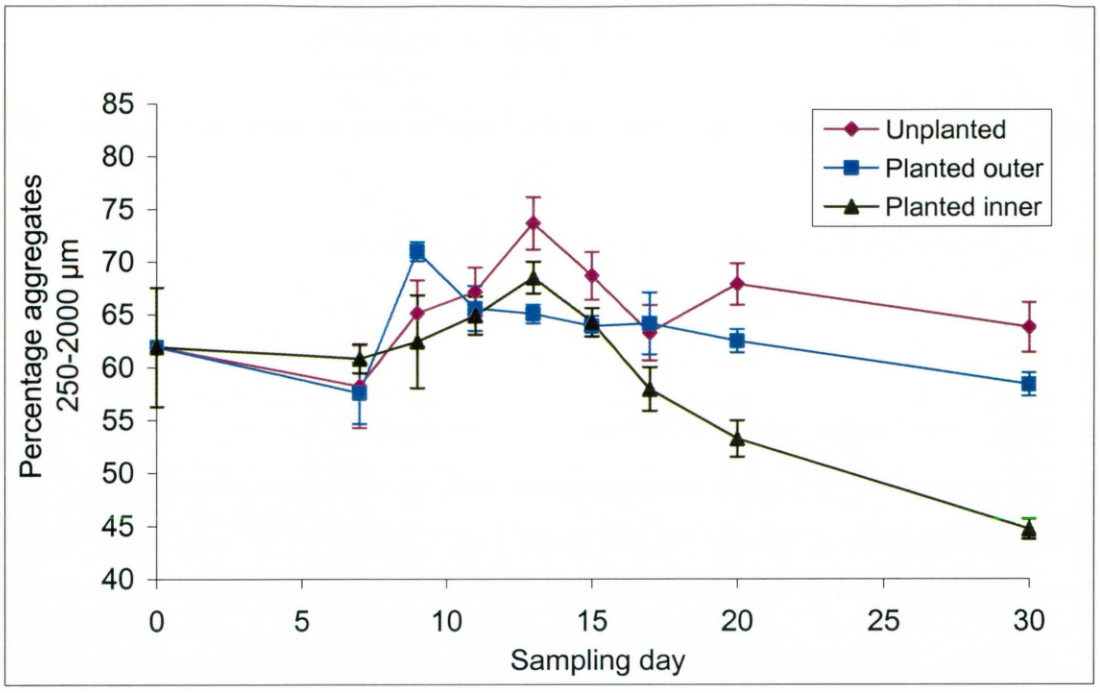


Fig. 3.7c Bullion Field soil, incubation and treatment effects upon percentage of aggregates 250-2000 µm (error bars indicate standard error of the mean).

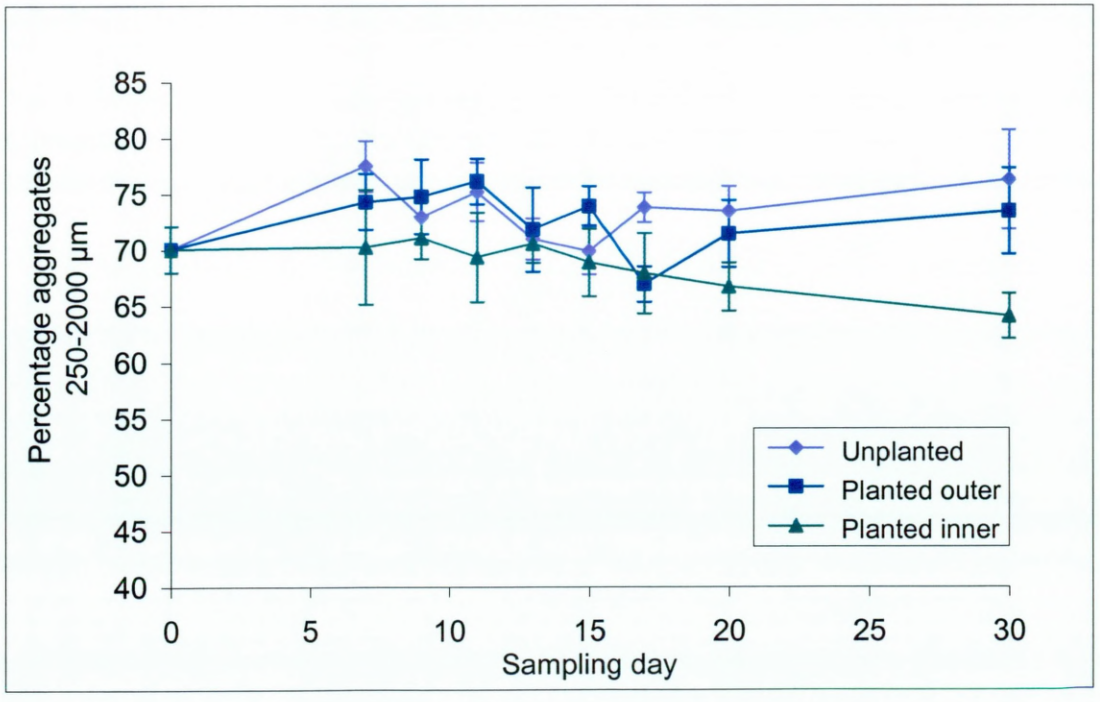


Fig. 3.7d Lab Field soil, incubation and treatment effects upon percentage of aggregates 250-2000 µm (error bars indicate standard error of the mean).

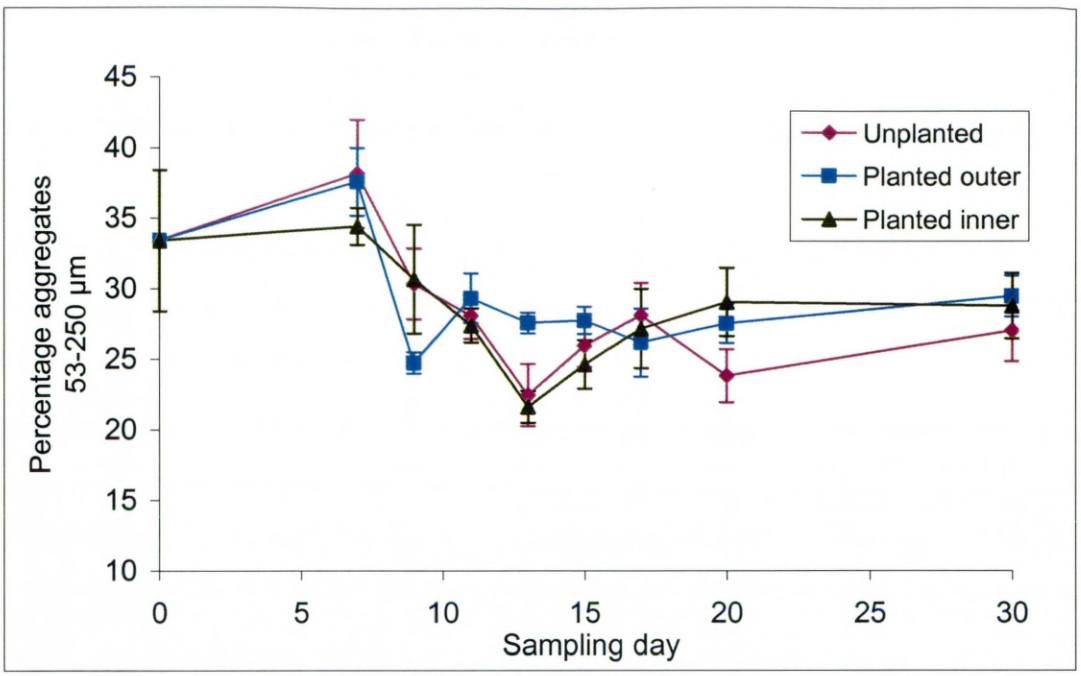


Fig. 3.7e Bullion Field soil, incubation and treatment effects upon percentage of microaggregates 53-250 µm (error bars indicate standard error of the mean).

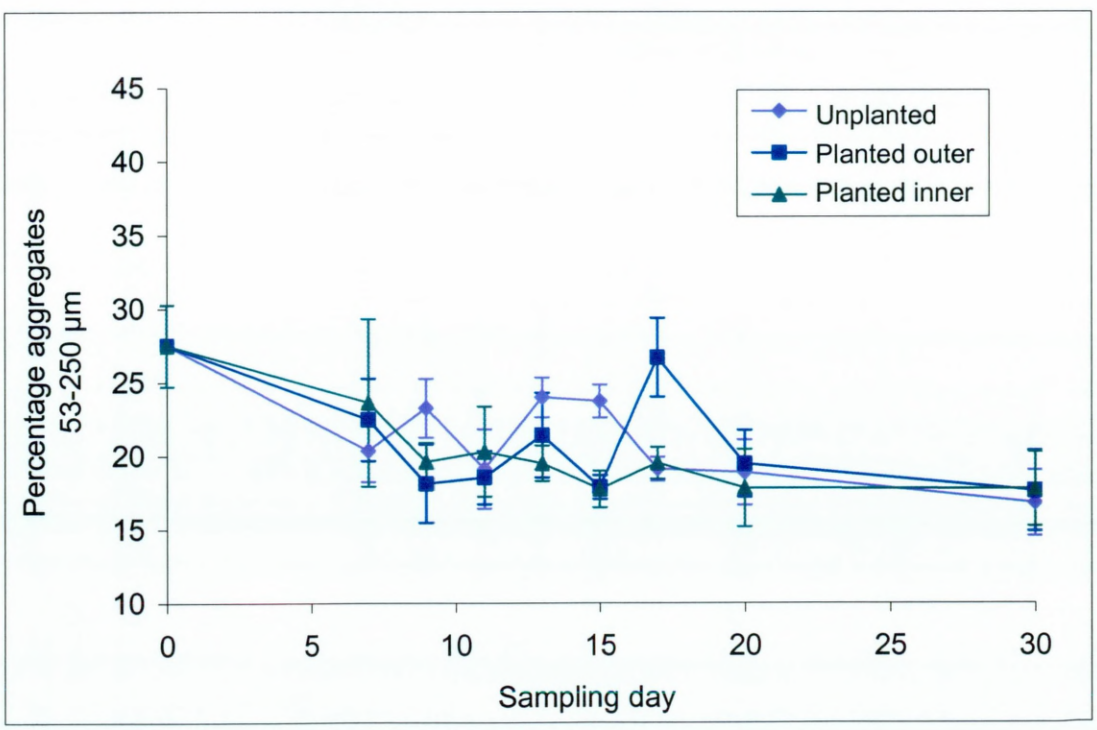


Fig. 3.7f Lab Field soil, incubation and treatment effects upon percentage of aggregates 53-250 µm (error bars indicate standard error of the mean).

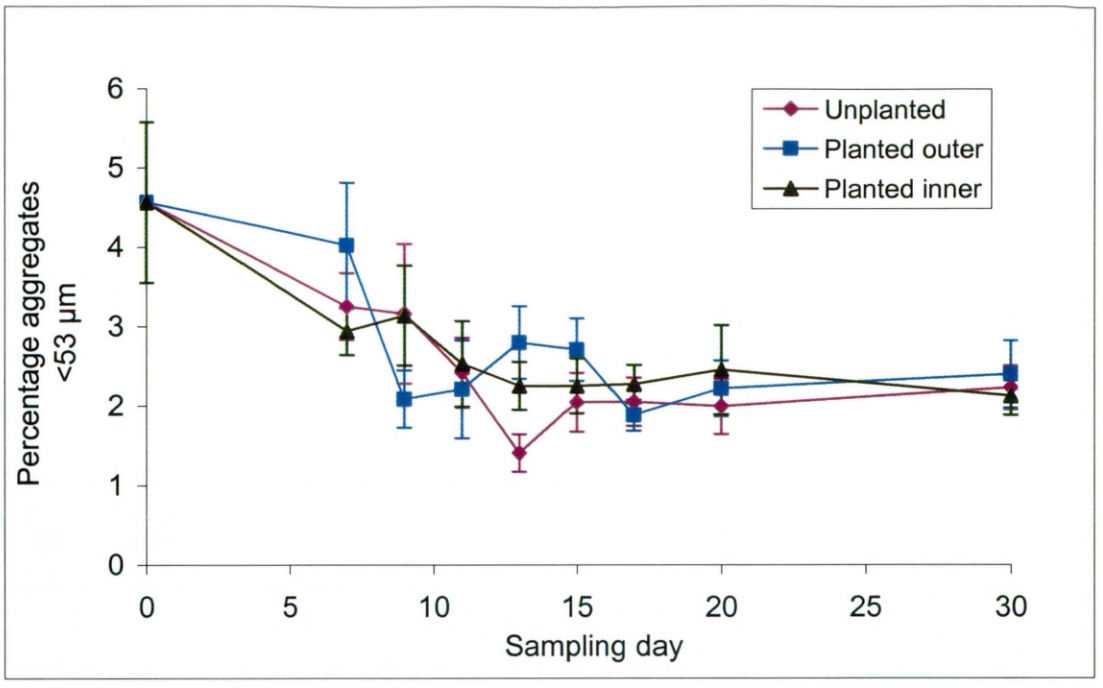


Fig. 3.7g Bullion Field soil, incubation and treatment effects upon percentage of fine particles or aggregates <53 µm (error bars indicate standard error of the mean).

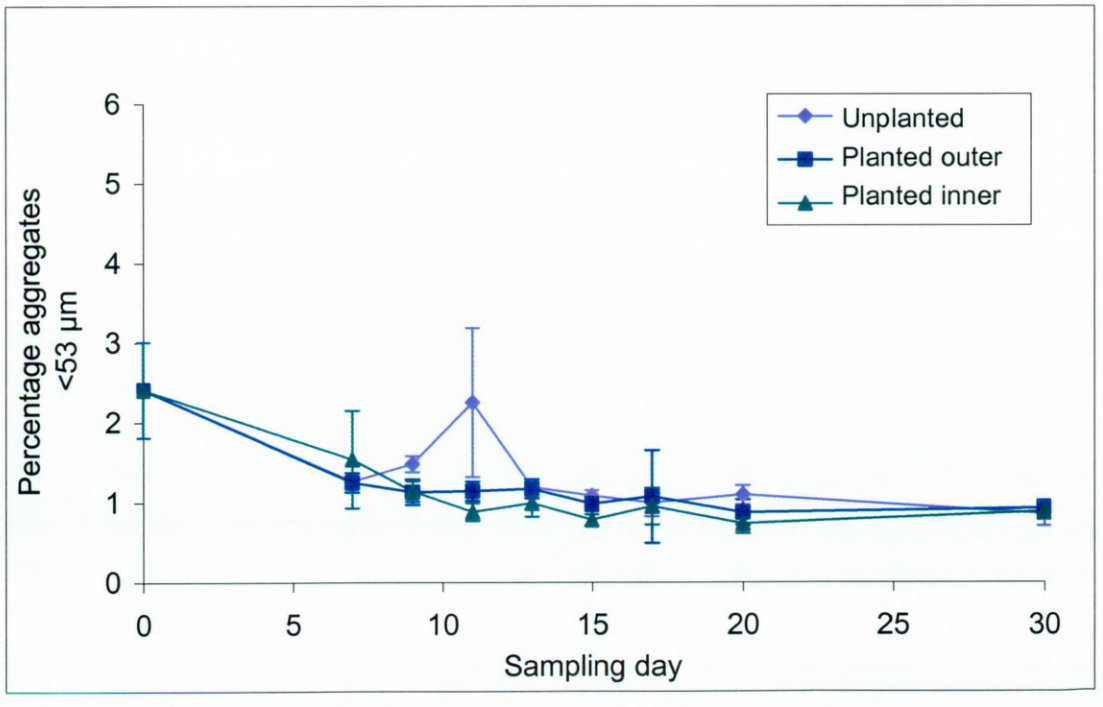


Fig. 3.7h Lab Field soil, incubation and treatment effects upon percentage of fine particles or aggregates <53 µm (error bars indicate standard error of the mean).

To illustrate the possible role of aggregate hierarchy upon active re-aggregation throughout the incubatory period samples from zero days incubation and 30 days incubation were plotted cumulatively (as illustrated in Fig 3.8a&b). The increasing proportion of aggregates >2000 μm is matched with a decline in the proportion of aggregates 250-2000 μm confirming the active binding of smaller structures into larger aggregates.

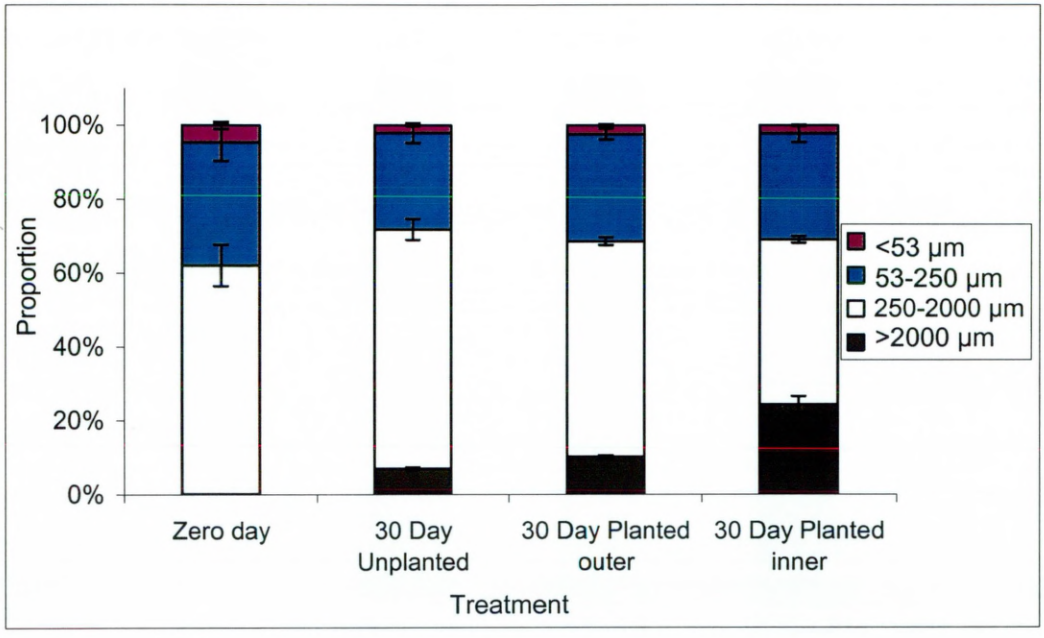


Fig. 3.8a Bullion Field soil cumulative aggregate breakdown of zero day and thirty day incubation treatments.

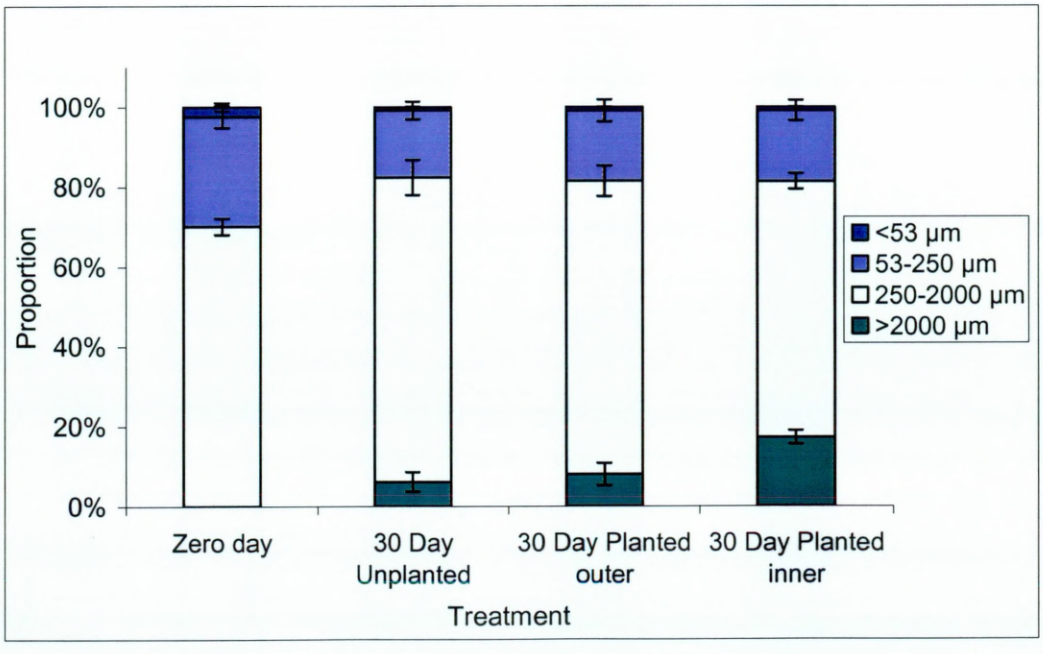


Fig. 3.8b Lab Field soil cumulative aggregate breakdown of zero day and thirty day incubation treatments.

3.3.6 Correlations between fungal biomass and water repellency

Trends between measures of ergosterol and water repellency are illustrated in plots shown in Fig. 3.9a-f.

For each treatment a Spearman rank correlation detected a significant positive correlation between fungal biomass and water repellency ($P<0.001$ for all treatments) under both experimental soils. The Pearson product-moment correlation (r) values for Bullion Field soils were: unplanted $r=0.697$, planted outer $r=0.588$, planted inner $r=0.666$.

The Pearson product-moment correlation values for Lab Field were: unplanted $r=0.498$; planted outer $r=0.464$; planted inner $r=0.532$ ($P<0.001$).

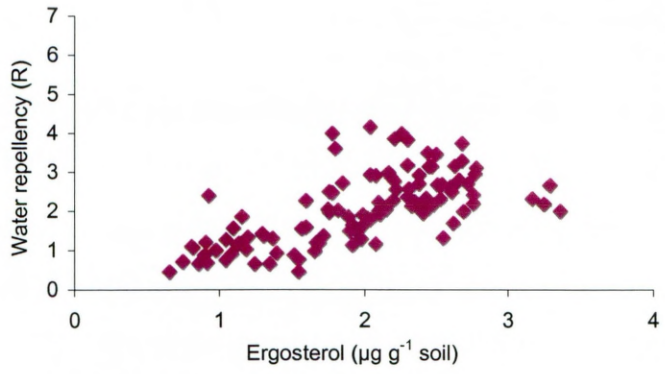


Fig. 3.9a

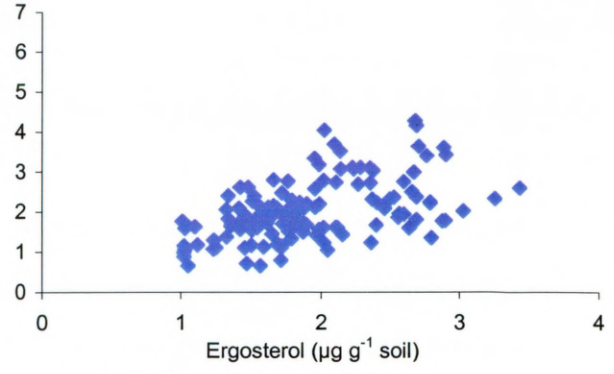


Fig. 3.9b

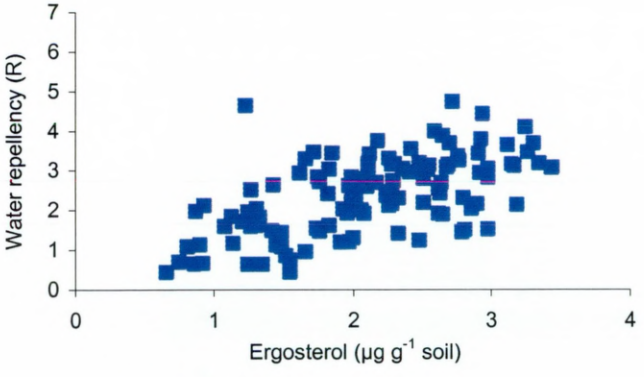


Fig. 3.9c

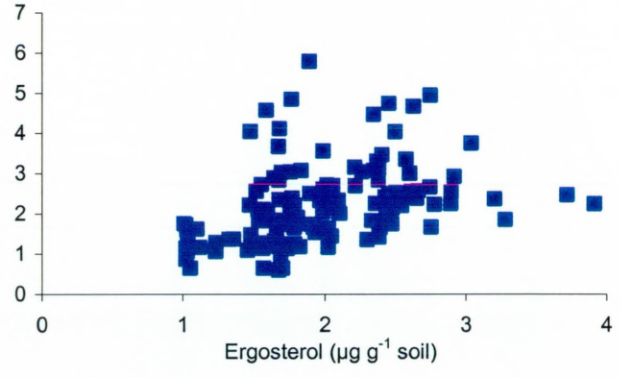


Fig. 3.9d

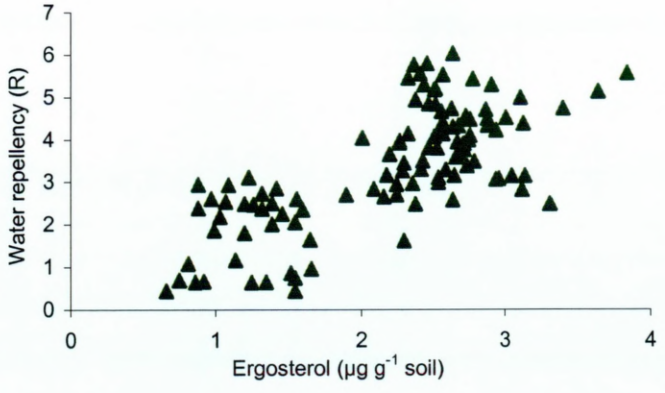


Fig. 3.9e

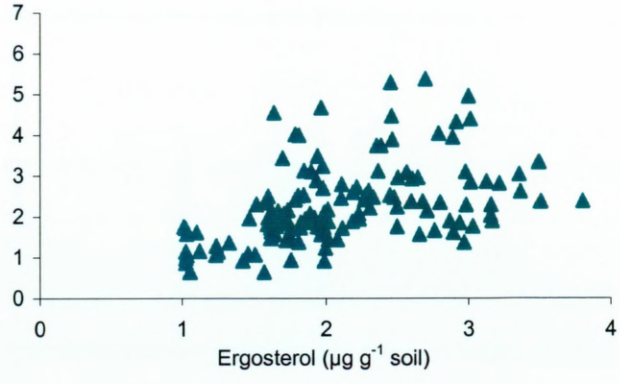


Fig. 3.9f

Fig. 3.9a-f Correlations between ergosterol concentration and water repellency, a: Bullion Field unplanted; b: Lab Field unplanted; c: Bullion Field planted outer; d: Lab Field planted outer; e: Bullion Field planted inner & f: Lab Field planter inner.

3.3.7 Correlations between glomalin and water repellency

The relationship between glomalin concentration and water repellency for Bullion Field soils is illustrated in Fig 3.10a-c. The correlation between glomalin and water repellency was considerably weaker than the correlation between ergosterol and water repellency. Under unplanted and planted outer treatments the Pearson product moment correlation (r) values were 0.293 and 0.222, respectively ($P<0.01$). Under the planted inner treatment there was a stronger relationship with a correlation of 0.506 ($P<0.01$), which may have been due to slightly higher levels of glomalin detected.

Under the Lab Field experimental soils no correlation between glomalin and water repellency was detected in any of the treatments unplanted $r=-0.034$; planted outer $r=0.105$; planted inner $r=0.12$, all $P>0.05$ (no illustrative plots are shown).

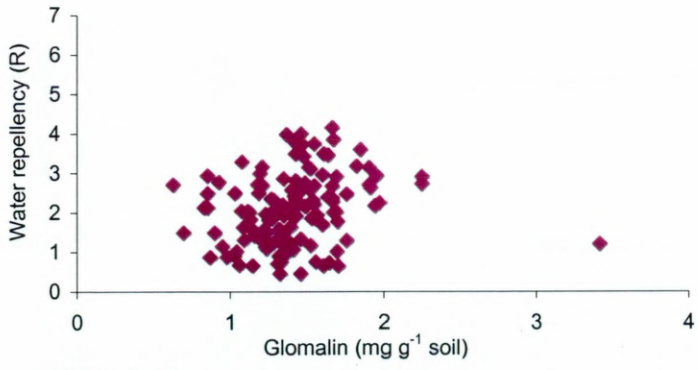


Fig. 3.10a

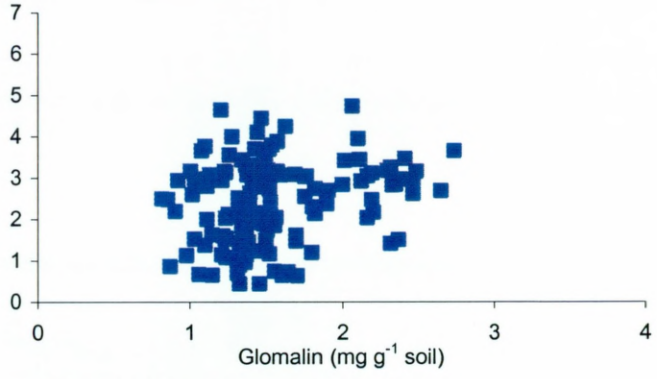


Fig. 3.10b

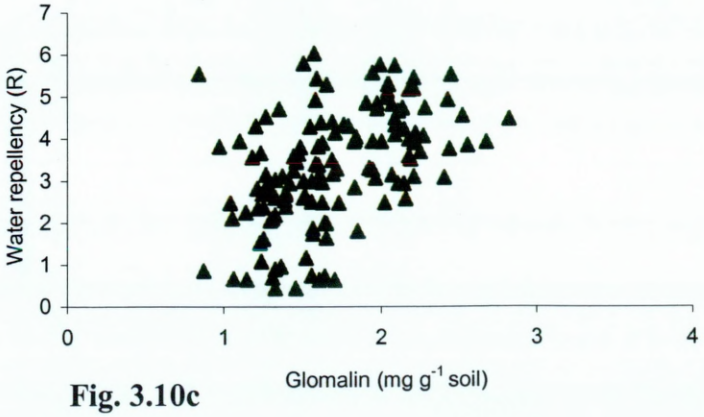


Fig. 3.10c

Fig. 3.10a-c Bullion Field soil correlations between glomalin and water repellency, a: unplanted; b: planted outer & c: planted inner.

3.3.8 Relationship between ergosterol and glomalin

Under Bullion Field soil positive correlations were detected under unplanted and planted outer treatments; the correlations between ergosterol and glomalin were 0.234 and 0.261 respectively ($P<0.01$), the correlation strengthened under planted inner treatments $r=0.470$, ($P<0.01$).

Under Lab Field soils the application of a Spearman rank correlation, did not detect any significant correlations between ergosterol and glomalin ($P>0.05$) under any of the treatments.

3.3.9 Relationships between aggregate size classes, water infiltration and fungal biomarkers

Bullion Field soil

In order to investigate relationships between soil measurements and various aggregate sizes a Spearman rank correlation was applied to the data, the results of which are shown in Table 3.2a-c. Under all treatments the percentage of >2000 μm aggregates was positively correlated with measures of ergosterol, glomalin and water repellency. The weakest correlation between percentage >2000 μm aggregates, glomalin and ergosterol was detected under the unplanted treatment. However, the strongest relationship between water repellency and aggregates of this size was also detected under the unplanted treatment. At smaller aggregate sizes few correlations with other measurements were detected, significant negative correlations were detected between percentage aggregates <53 μm and water repellency but only under the unplanted and planted inner treatments.

Measure	>2000 μm	250-2000 μm	53-250 μm	<53 μm
Ergosterol	**0.435**	0.033	0.196	0.196
Glomalin	**0.348**	-0.107	-0.065	0.046
Repellency	**0.940**	0.013	0.077	**-0.331**

Table 3.2a Bullion Field soil, unplanted.

Measure	>2000 μm	250-2000 μm	53-250 μm	<53 μm
Ergosterol	**0.706**	-0.237	0.132	0.132
Glomalin	**0.676**	**-0.348**	-0.012	-0.012
Repellency	**0.792**	**-0.409**	0.200	0.103

Table 3.2b Bullion Field soil, planted outer.

Measure	>2000 μm	250-2000 μm	53-250 μm	<53 μm
Ergosterol	**0.865**	-0.223	**-0.353**	-0.212
Glomalin	**0.668**	-0.198	-0.094	-0.199
Repellency	**0.758**	-0.045	-0.353	**-0.388**

Table 3.2c Bullion Field soil, planted inner.

Tables 3.2a-c Bullion Field soil Pearson product-moment correlation values (*r*) assessing relationships between aggregate sizes and other measurements. Values in bold indicate correlations that are significant to $P<0.05$.

Lab Field soil

The results of a Spearman rank correlation applied to the data are shown in Table 3.3a-c. Similarly to the previous soil the majority of positive correlations were detected at the >2000 μm scale. Glomalin was correlated with the percentage >2000 μm aggregates under the planted inner treatment only, no correlation under other treatments was detected. Water repellency was not correlated with percentage aggregates >2000 μm under the unplanted treatment, this was not the case for other treatments or the previous soil. At other aggregate sizes any other significant relationship detected was a negative correlation, in particular the proportion of aggregates <53 μm was negatively correlated with ergosterol under all treatments.

Measure	>2000 μm	250-2000 μm	53-250 μm	<53 μm
Ergosterol	**0.456**	0.214	**-0.475**	**-0.429**
Glomalin	-0.217	0.106	-0.019	-0.043
Repellency	0.266	**0.296**	**-0.471**	**-0.354**

Table 3.3a Lab Field soil, unplanted.

Measure	>2000 μm	250-2000 μm	53-250 μm	<53 μm
Ergosterol	**0.486**	0.065	-0.286	**-0.400**
Glomalin	0.081	-0.188	0.091	**-0.309**
Repellency	**0.516**	0.034	-0.240	0.114

Table 3.3b Lab Field soil, planted outer.

Measure	>2000 μm	250-2000 μm	53-250 μm	<53 μm
Ergosterol	**0.628**	**-0.307**	-0.250	**-0.414**
Glomalin	**0.301**	-0.138	-0.103	-0.219
Repellency	**0.434**	0.001	**-0.304**	**-0.361**

Table 3.3c Lab Field soil, planted inner.

Tables 3.3a-c Lab Field soil Pearson product-moment correlation values (*r*) assessing relationships between aggregate sizes and other measurements. Values in bold indicate correlations that are significant to $P<0.05$.

3.3.10 Lab Field soil: Microaggregate size distribution

Seven samples from the Lab Field experimental cores were selected for microaggregate size analysis; the resulting microaggregate size distribution is displayed in Fig. 3.11. No radical changes in microaggregate size distribution were apparent. To detect for differences a Kruskal-Wallis test was applied on a breakdown of soil size classes: 100-50, 50-20, 20-10, 10-5, 5-2 and >2 μm. The resulting *P*-values are displayed in Table 3.4, and show significant differences between two or more of the groups at all size classes with the exception of 100-50 μm. The size class containing the greatest proportion of soil was 50-20 μm, at this scale the zero day sample had the smallest proportion of soil in this class and the largest proportion was detected under the 30 day outer planted samples. The

differences detected were however very subtle, as other size fractions showed no distinct trends detectable with varying samples having the highest and lowest proportions in each group.

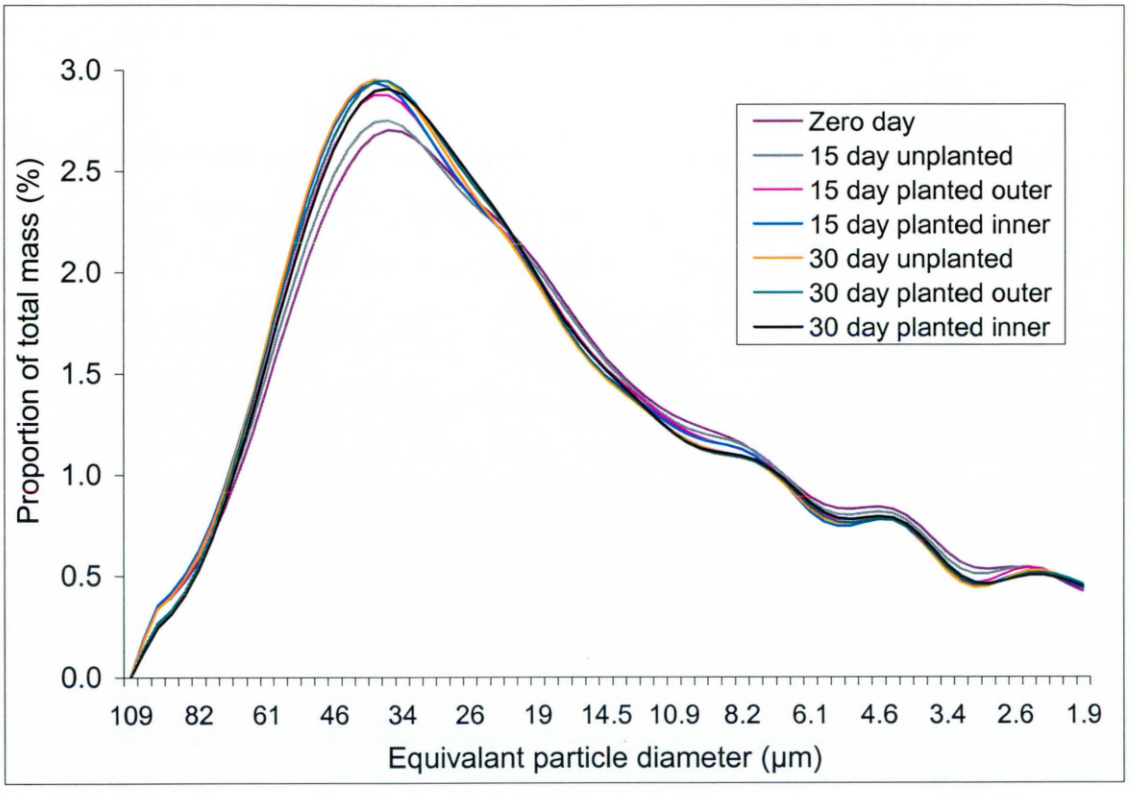


Fig. 3.11 Resulting microaggregate size distribution for Lab Field soil samples, with each line representing the mean of five replicate readings.

Size Fraction	P-Value
100-50 µm	0.305
50-20 µm	**0.000**
20-10 µm	**0.045**
10-5 µm	**0.001**
5-2 µm	**0.000**
>2 µm	**0.000**

Table 3.4 Lab Field soil microaggregate size fraction breakdown, *P*-values indicating the level of significance of differences between different treatments.

3.4 Discussion

The aims of this chapter were to assess the impact of fungi (with and without the presence of roots) upon water repellency and subsequently their relationship to soil structural stability.

The initial breakdown of soil to <2000 μm was unlike previous authors (Bossuyt *et al.*, 2001; Denef, *et al.*, 2001b), who subjected the soil to radical sieving (<250 μm) whereby large quantities of organic matter were removed from the soil. The experimental set-up presented was designed to disturb the soil without completely destroying the structure, and examine if fungi bind aggregates to >2000 μm. The experimental set-up demonstrated that throughout the incubatory period, aggregates <2000 μm appeared to become actively bound into aggregates >2000 μm under all treatments (illustrated in Fig. 3.8), a factor also reported by Tisdall and Oades (1979).

Disruption of the test soils (by sieving to <2000 μm) initially resulted in the destruction of aggregates >2000 μm. In both experimental soils there was a significant recovery and reformation of >2000 μm aggregates throughout the experimental period. Within Bullion Field soil there were a greater proportion of aggregates of this scale in comparison to Lab Field, perhaps reflecting the more stable structure of Bullion Field. Changes in other aggregate size classes were not as clearly marked. Aggregates of 250-2000 μm for both soils showed a general decline for planted inner treatments, indicating that aggregates of this size were actively being bound into aggregates >2000 μm. This was not the case, however, for aggregates 53-250 μm as no detectable trend was apparent for either soil. Both soils showed a marked decrease in aggregates or fine particles >53 μm under all treatments.

The measurement of microaggregate size distribution (<109 μm) quantified using X-ray sedimentation was completed upon Lab Field soil. Microaggregate size distribution at this scale differed subtly between the time points. There were

significant differences between treatments detected under various size breakdowns, however the general trend in size distribution did not change radically between treatments. One of the downfalls of X-ray sedimentation could be the nature of the sample preparation. Samples were effectively forced through a 109 μm sieve, so the energy used may differ from sample to sample and could result in fine changes in size distribution. Also the suspension of the samples was in this case done by hand, another factor that may result in variability. There may have been no impact of treatment upon particle size distribution at less than 100 μm.

In the structurally unstable soil (Lab Field) glomalin was correlated (weakly) with the proportion of aggregates >2000 μm only under the planted inner treatment, showing no correlations under other treatments. Under Bullion Field soil, however, there were correlations between aggregates >2000 μm and glomalin under all treatments. A weaker correlation was detected under the unplanted treatment with the level of correlation increasing under the planted outer and inner treatments respectively. Greater concentrations of glomalin were detected under Bullion Field soil, which also had higher root length colonization than Lab Field. These factors, along with the higher structural stability of Bullion Field, may explain the significant relationship detected. Prior to incubation neither soil had a relationship between glomalin and aggregate stability. Both soils were agricultural soils with moderate levels of glomalin. The development of a relationship following incubation indicates that a certain level of glomalin may be required before a relationship is detected.

Ergosterol was highly correlated with the proportion of aggregates >2000 μm for both experimental soils. Ergosterol was negatively correlated with the proportion of aggregates <53 μm for Lab Field soil but not Bullion Field soil. The detection of a relationship between ergosterol and aggregates >2000 μm under all treatments is indicative that even without the binding influence of roots there was a significant influence of fungal hyphae upon aggregation of soil. Andrade *et al.* (1998) reported that hyphae contribute to soil structure stabilisation independently of plant root contributions. Similarly to the results presented here, Jastrow *et al.* (1998) also reported that the strongest correlation of binding agents in aggregates >2000 μm was fungal hyphae. Jastrow *et al.* (1998) however, studied only mycorrhizal fungi and

their investigation used field based samples, thus did not isolate the effects of roots. Ergosterol is an indicator of total fungal biomass and not specifically AM fungi, the correlation detected under the unplanted treatment is indicative that without the presence of roots and AM fungal hyphae, other soil fungi are capable of significantly stabilising soil structures. The increased fungal biomass in unplanted treatments indicated that saprophytic fungi were present in considerable numbers; this was likely to be a disturbance effect due to the initial release of previously inaccessible organic matter after the process of sieving. To allow the investigation of a soil containing predominantly AM fungi, future experimental soils could be sieved and allowed to pre-incubate. This process would let the flourish of saprophytic fungal activity to occur prior to experimental perturbations, and allow the AM fungal inoculum to germinate with minimal competition.

Both soils had a positive correlation between fungal biomass and water repellency, a relationship similarly reported by White *et al.* (2000) who reported increased hydrophobicity in the presence of fungi. The strongest relationship was observed in Bullion Field soil, even though both soils reached similar levels of ergosterol concentration. However, water repellency was considerably higher in Bullion Field soil (4.8) compared to Lab Field (3.0). This may indicate a difference between the two experimental soils, and could plausibly have been caused by the increased glomalin concentration detected under Bullion Field soil. The changing soil water repellency levels throughout the incubatory period may be attributed to the influence of fungi, through changes in hydrophobicity. Ethanol sorptivity did not differ throughout the incubatory period (data not shown) indicating that there were limited changes in the pore structure. This, however, does not give an indication of micro-scale pore changes in the pore network, which may have influenced water repellency, and this is something that would merit further investigation.

Glomalin was not correlated with water repellency in any of the treatments in the Lab Field soil. Glomalin did, however, show a positive correlation with water repellency in Bullion Field soil but only under the planted inner treatment. Under this treatment, the highest concentration of glomalin was detected, as was the highest level of water repellency. The increased concentration of glomalin detected under

Bullion Field soil might be due to a higher carbon and nitrogen levels than the Lab Field soil (Table 3.1), this difference may stimulate increased microbial exudate production (Auer and Seviour, 1990) and may also explain the increased water repellency in Bullion Field soils. Initially it was reported that glomalin might be an indicator of AM fungal biomass, although more recent publications dispute this (Rillig and Steinberg, 2002; Rillig *et al.*, 2001; Steinberg and Rillig, 2003). The only correlation between glomalin and ergosterol was detected under Bullion Field soil. Under unplanted and planted outer treatments relatively weak correlations were detected with the strongest relationship detected in the planted inner treatment. Higher concentrations of glomalin were detected under Bullion Field soil with the strongest concentration found under the planted inner treatment. These differences are perhaps indicative of a threshold level of glomalin required before a relationship is detected.

Caron *et al.* (1996) and Guggenberger *et al.* (1999b) hypothesized that changes in soil wetting were likely to be the causal factor in the stabilisation of soil. In the experimental set-up water repellency was positively correlated with aggregates >2000 μm. This result may confirm the mechanism of aggregate stabilisation proposed. Additionally, the positively correlated increases in fungal biomass are indicative that biologically mediated changes in water relations are likely to be one of the key mechanisms involved in aggregate stabilisation. Thus, measures of water infiltration may be a more suitable measure of soil stability by directly measuring the mechanism responsible.

No measure of root length (per gram of soil) was made; therefore the direct impact of roots can only be hypothesised. Allison (1968) investigated the impact of grass roots upon aggregate stabilisation, suggesting that pressures from roots directly influence aggregation as does the removal of water, which induces localised drying, whilst fine roots act as a source of organic matter. The localised drying of soil reported by Allison (1968) may have been responsible for changes in soil water repellency. Oades and Waters (1991) suggested that roots and hyphae act together as a "sticky string bag" creating a web-like framework, initially stabilising aggregates. This process may actively encourage other microbes within aggregated soil, a

process also reported by Foster (1988), Jastrow and Miller (1998), Jastrow *et al.* (1998) and Oades and Waters (1991). The physical entanglement of soil particles by roots, especially those that produce complex roots systems (such as grasses), was reported by Jastrow *et al.* (1998). They reported that roots may act both directly and indirectly in soil stabilisation (see Fig 1.2, Section 1.2.3). They separated the influences of very fine roots (those <0.2 mm in diameter) and fine roots (0.2-1 mm in diameter) reporting that very fine roots were responsible for enmeshment, whilst fine roots were more important for their associations with AM fungi.

The results presented show that in the short time-scale of 30 days significant increases in aggregation were detected under all treatments. Further more detailed measures of the complex interactions involved are required; Jastrow *et al.* (1998) reported in their work that the covariance between binding agents mean that a "*simple comparison of correlations could lead to erroneous conclusions*" of the impact of particular binding agents, a factor that should be taken into account upon further work.

3.5 Conclusions

The experimental set-up used two arable soils. One soil was prone to slaking (Lab Field), whilst the other was considered structurally stable (Bullion Field). The aim of the experiment was to investigate temporal changes in fungal biomass, and how these changes relate to water relations and subsequently aggregate stabilisation, across two contrasting soil ecosystems.

The two soils demonstrated similar trends in most of the measurements, with both soils showing increases in fungal biomass, water repellency and large macroaggregates (>2000 μm) throughout the incubatory period. However, the extent to which these measurements increased differed between soils, with lower levels of fungal biomass, repellency and large macroaggregates (>2000 μm) in the Lab Field soil. Fungal biomass was shown to have a significant positive correlation with water repellency, and water repellency was positively related to the proportion of

aggregates > 2000 μm. The results indicate that in addition to the binding influence of fungal hyphae there is significant proof of a change in the wetting behaviour of the soil by fungi (and roots). The wetting behaviour of the soil may be a more appropriate assessment of soil stability than aggregate size fractioning.

Further work should be completed to establish whether roots and fungal hyphae change soil-wetting behaviour as a result of internal soil aggregate micro-scale structural changes, and or through the deposition of hydrophobic compounds. Work of this nature would allow a greater understanding of soil physical and biological interactions.

Chapter 4: Visualising changing soil structure at the micro scale, a three dimensional investigation of soil aggregates

4.1 Introduction

Structural investigations of undisturbed soil are important to establish how changes in properties relating to soil stability (such as aggregate stability and soil water infiltration) are linked to soil architecture. The assessment of soil pore space is vital, as the volume and connectivity of pores is a regulatory factor in all soil biological, physical and chemical processes (Pierret *et al.*, 2002).

Various soil processes occur at different pore scales. Macro-pores are considered to be >1000 μm diameter and are associated with bypass flow (Luxmoore, 1981). Luxmoore (1981) proposed various pore size classes and reported associated soil water processes, which are displayed in Table 4.1.

Porosity class	Pore diameter	Associated processes
Micro	<10 μm	Evapotranspiration; matric pressure gradient for water redistribution
Meso	10-1000 μm	Drainage; hysteresis; gravitational driving force for water dynamics

Table 4.1 Pore scales and associated soil water processes, (based on Luxmoore, 1981).

McKeague *et al.* (1982) suggested that studies of soil porosity are restricted by the lack of appropriate measurement techniques. One possible technique to examine the internal architecture of soils is X-ray computed tomography (CT), which was used by Crestana *et al.* (1985) to observe water content and movement within soil. However, Crestana *et al.* (1985) did not measure porosity or present any quantifiable differences in structure, but proposed that CT scanning would have great potential in soil science. Jègou *et al.* (2002) and Pierret *et al.* (2002) investigated soil porosity and pore connectivity through the application of CT scanning. These investigations studied macro-porosity, observing the soil at resolutions of 0.5-3 mm, often assessing the impact of earthworm activity upon soil structure (Jègou *et al.*, 2002; Pierret *et al.*, 2002).

Rasiah and Aylmore (1998) assessed the impact of wetting and drying upon porosity, pore continuity and the spatial distribution of pores. They attempted to use CT as a reliable estimation of unsaturated hydraulic conductivity, but did not utilise the methodology to assess how experimental perturbations impacted upon soil properties.

Soil tomographical investigations have progressed rapidly during the past decade (Crestana *et al.*, 1985; Hainsworth and Aylmore, 1983; Heijs *et al.*, 1995; Macedo *et al.*, 1999; Phogat and Aylmore, 1989; Warner *et al.*, 1989) but to my knowledge no attempt has been made to link physical changes in soil structure to microbial or plant root activity. Investigations of this nature would need to be carried out at a resolution of <10 μm in order to link changes in soil structure to that of microbial activity and activity associated with very fine plant roots.

The aim of this chapter is to measure and analyse differences in soil microstructure using X-ray computed tomography. Significant differences between treatments have already been detected at scales ≥2000 μm (Section 3.3.5), with increases in the proportion of macroaggregates and water repellency after thirty days incubation. These changes in macro-scale structure were related to fungal and plant activity; the aim of the work presented in this chapter is to assess how changes in fungal biomass, and changes at the macro-scale will be directly related to changes detected at the micro-scale. Utilising a micro-tomographical source from synchrotron radiation, the impact of the treatments outlined in Section 3.2.1 upon micro-scale structure (100-4 μm) is investigated.

4.2 Materials and methods

4.2.1 Samples scanned

Aggregates from the experimental set-up presented in Chapter 3 were selected for analysis. Samples from the Lab Field investigation were assessed (Sections 3.2 & 3.3.5). Five replicate air-dried aggregates of >2 mm in diameter from each of the following treatments were randomly selected for analysis: 30 days incubation unplanted, planted outer (no roots), planted inner (with roots) and zero days incubation. This gave a total of 20 scanned aggregates (for more comprehensive details on sample treatments see Section 3.2.1). Slight pressure was applied to the >2 mm soil aggregates resulting in smaller aggregates (<2 mm), which were used for scanning. The pressure applied to the aggregates was minimal and was designed to split the soil aggregates at weaknesses in the structure, thus limiting the impact upon internal pore structure. This procedure was applied to soil aggregates from all treatments.

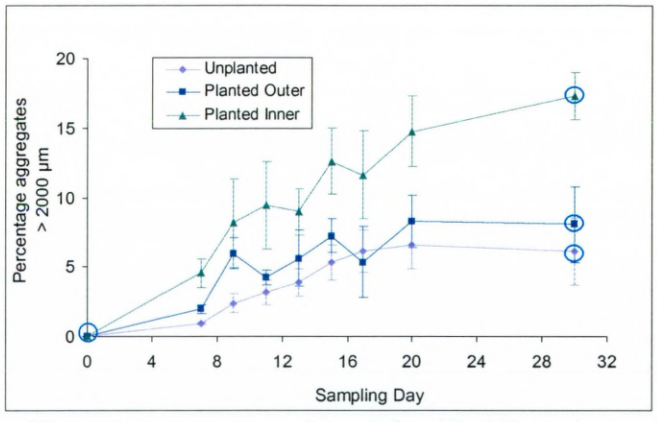


Fig. 4.1 Aggregates selected for 3D CT analysis, highlighted samples illustrate those selected from experimental set-up described in Section 3.2.1.

4.2.2 Scanning procedure

The samples were X-ray scanned at the Advanced Photon Source (APS) within the Argonne National Laboratory (Illinois, USA) in the GeoSoilEnviro Centre for Advanced Radiation Studies (GSECARS, station 13-BM-D). Scanning was processed using X-rays at 7 GeV. Samples were analysed at 360 projections and were exposed for 4 seconds per projection, the resulting 360 X-ray images were used for image reconstruction. Prior to image reconstruction pre-processing was completed using software written in Interactive Data Language (IDL) to correct for dark and flat-fields. Reconstruction of images was completed using a Fourier

Transform algorithm, supplied by GSECARS (http://cars9.uchicago.edu/software/idl/tomography.html).

Reconstruction of images resulted in a sequence of approximately 650 image slices (650 × 515 pixels) with a resolution of 4.4 μm. The set-up used to capture sample images including the rotating sample stage, microscope and camera are shown in Fig. 4.2.

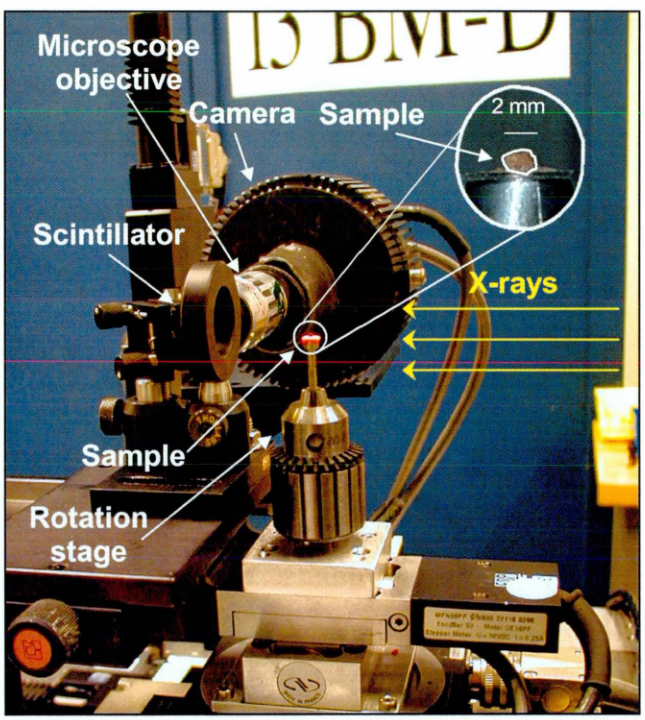


Fig. 4.2 Rotating sample platform for X-ray measurements of soil aggregates, showing camera used for image capture (image courtesy of Professor Karl Ritz).

4.2.3 Image cleaning and segmentation

Digital images are represented as a collection of discrete (and usually small) cells, which are known as pixels (picture elements). The initial images of the scanned samples were in a grey scale format, whereby each pixel is a shade of grey and has a single numerical value related to brightness ranging from 0 (black) to 255 (white). The cleaning and segmentation of images was completed on the basis of the various pixel values, allowing the segmentation of image sets into soil aggregate, inter-aggregate pore and surrounding void.

All image processing and analysis was completed using KS300 software (Zeiss). A procedure was written (by Dr Naoise Nunan, Plant Soil Interface/BioSS, SCRI) to segment images into pore and solid spaces for subsequent statistical analysis. Image segmentation divides an image into regions of interest (dark or bright regions for example) and background regions that are not of interest. This is generally achieved by setting a threshold greyscale value; pixels with greyscale values greater or smaller than the threshold are selected for analysis. In this instance such an approach was not possible as the distinction between solid and void space was not always clear due to background noise (highlighted in Image 1, Fig. 4.3b). Therefore, a number of processing steps were required prior to image segmentation. A flow chart of the processing steps and example images are shown in Figs. 4.3a&b. The first processing step was a reduction in the greyscale range. This step reduced the brightness of very bright features in the image and was necessary because bright features caused darker features in their vicinity to be lost at the segmentation stage. Two smoothing steps were applied to the images using a Sigma filter (Fig. 4.3b, Image 2). Sigma filters reduce noise but preserve fine structures within the image.

Smoothed images were then segmented to produce binary images that were used for analysis (Fig. 4.3b, Image 4). Segmentation of the smoothed image was carried out using an adaptive greyscale segmentation. The function subtracts a lowpass filter of the smoothed image from the smoothed image and segments the resultant image according to a defined threshold. The lowpass filter calculated the average greyscale value of all pixels within a box of a defined size (here, 255 × 255 pixels) around a central pixel and the greyscale value of that pixel was then replaced by the average value. Subtraction of the lowpass image from the smoothed image resulted in an image in which greyscale values represented the difference between a pixel and its local background (within a box of 255 × 255 pixels centred on the pixel). Segmentation of this image by setting a threshold value selected only those pixels that were brighter than the local background. In this case, a low threshold value was set (value: 7) to include as much soil aggregate material as possible. This meant that the binary image contained noise as well as the constituent parts of the soil aggregate.

Images were subjected to a further segmentation step, also with an adaptive function, setting the threshold at a higher level, the resulting binary images thus containing only the brightest features in the image, all of which were part of the aggregate. Binary morphological closing and filling operations were applied to remove gaps between features, with the resulting binary image containing a single object, which occupied the area of the soil aggregate (Image 4, Fig. 4.3b). This image was used as a mask separating the soil aggregate from surrounding external noise. Pixel noise within pore space was eliminated by removing small features (<5 pixels).

The nature of the cleaning and segmentation macro meant that some features of the aggregate were not selected as soil matrix, but grouped with pore space. These features, generally smaller and brighter than the void region, were detected and added to the soil matrix (Image 5, Fig. 4.3b). Finally, small features such as narrow cracks or pores were detected, using a valleys finding operation upon the original image and these were added to the binary image (Fig. 4.3b Image 6). Further binary scrapping and dilation steps ensured the final removal of pixel noise. Throughout this process the original image was used as a reference ensuring the accuracy of the cleaning and segmentation process. The final step was to separate intra-aggregate pores from the image background; this was completed by slightly eroding the internal pore system and applying a colour (grey) to the internal pores, so pore space could be differentiated from the surrounding void. Thus, the final image contained three regions distinguished by the grey scale value of their pixels; solid (white), internal pore (grey, greyscale value 123) and surrounding pore (black).

For each of the aggregate image sets 200 images were selected for analysis. The beginning and end images that contained more background than aggregate were discarded; images were selected on the basis that they contained the central region of the aggregates, reducing the influence of edge effects.

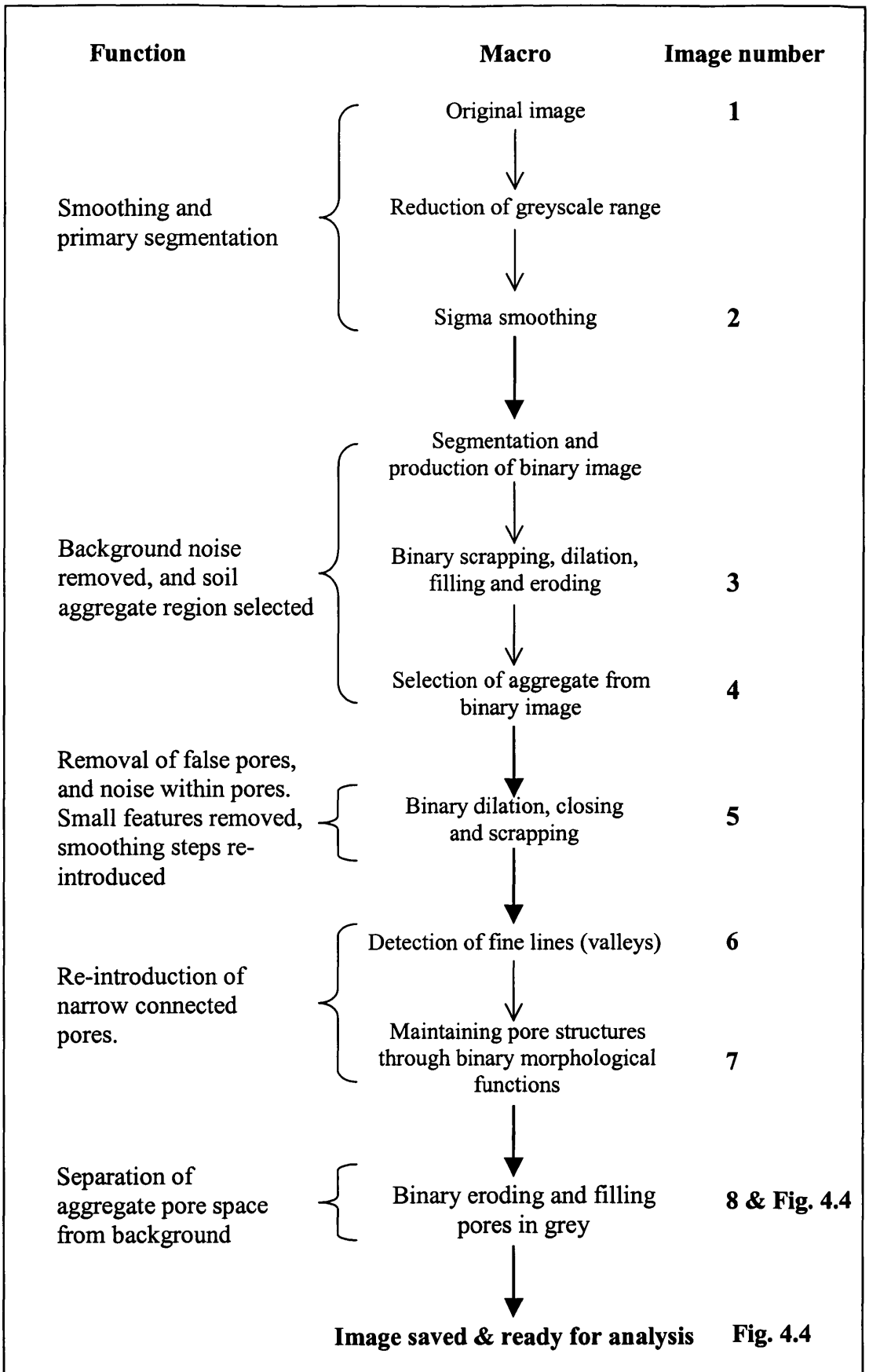


Fig. 4.3a Flow chart of image processing methodology applied to 3D CT digital images of soil aggregates.

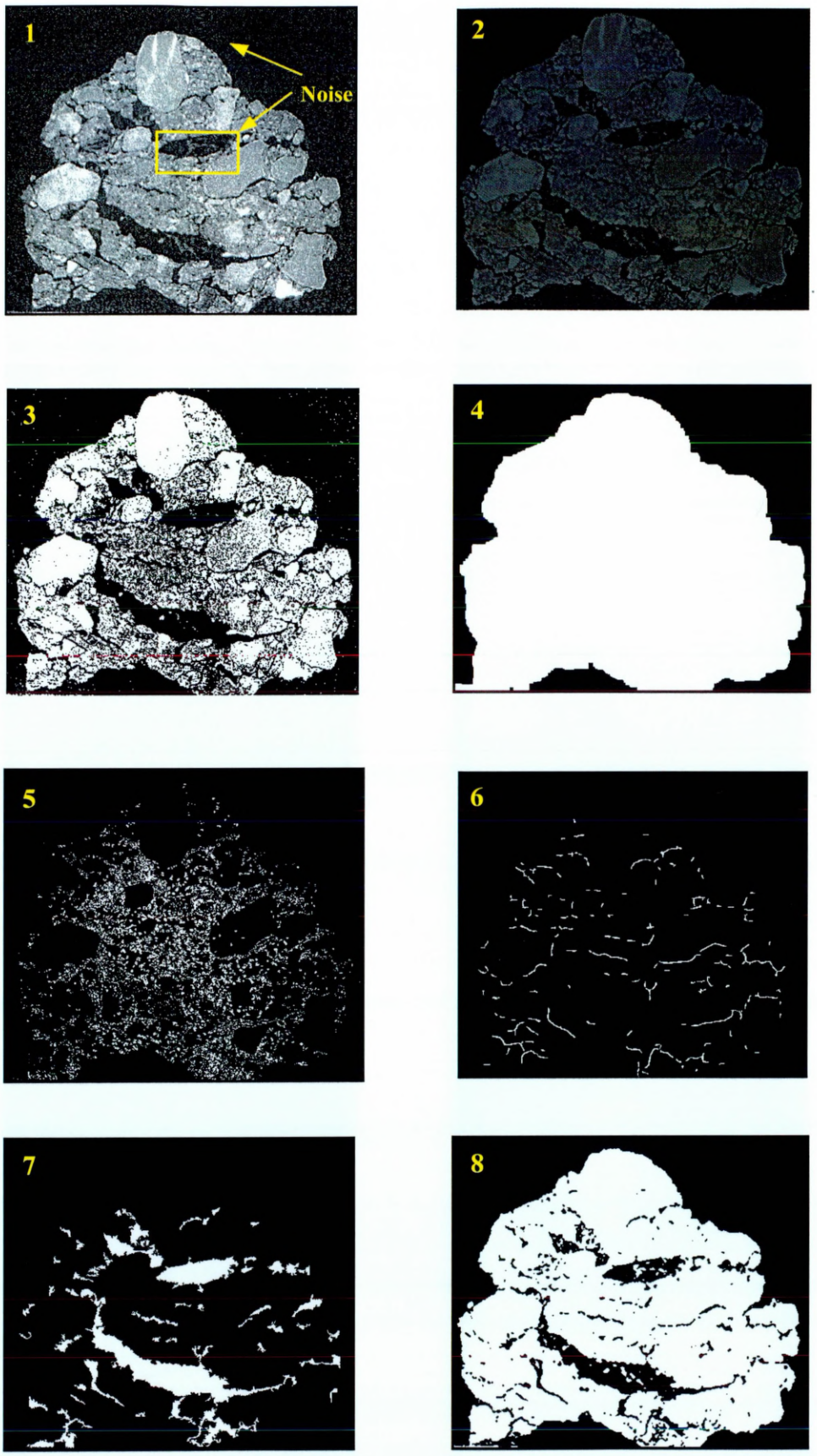


Fig. 4.3b Example images representing various steps of image cleaning and segmentation. Image noise is highlighted in image 1.

Cropped images were converted using XN-view public domain software (http://www.xnview.com/), into portable grey map files. Each of the image sets had a 200 × 200 pixel region selected from the centre of the image, avoiding as far as possible the edges of the soil aggregate (Fig. 4.4). This region was used for analysis. A data file (Text file) was extracted from the final binary image in which a value of 0 was given to pore features within aggregates; a value of 1 was given to solid matter and a blank value given to surrounding void pixels. This ensured sample volume consistency between and within treatments and avoided edge effects as far as possible. The stacked 200 single image files were converted into one file for analysis using a program written by Dr Jussi Sillanpaa (Scottish Crop Research Institute). This was completed for each of the aggregate image sets, and resulted in the data sets that contained 8 million values (~24 Mb) per aggregate.

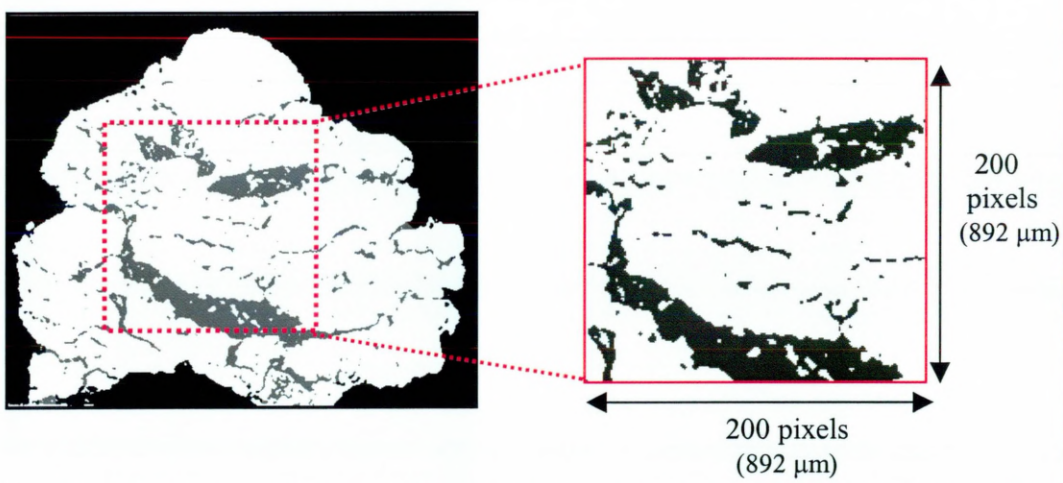


Fig. 4.4 Selection of 200 × 200 pixel region of 3D CT scanned image after cleaning and segmentation. Pore space grey, solid white and image background black, section for analysis highlighted.

4.2.4 Image and statistical analysis

Quantifying soil porosity

Using a macro written with KS300 software (Zeiss), the numbers of solid (white) and porous (grey) pixels were quantified for each of the image sets (containing 200 cropped images per aggregate image set). The percentage porosity was calculated:

$$\text{Porosity (\%)} = \frac{N^{os} \text{ grey pixels (pores)}}{N^{os} \text{ total pixels (grey pixels + white)}} \times 100 \quad (4.1)$$

Differences in porosity between treatments were statistically analysed using an ANOVA (Genstat, Release 6.1).

Spatial analysis of soil structures

Spatial correlations in aggregate data sets were analysed using semivariance analysis using the geovariance analysis software package Isatis 4.0 (Geovariances, Fontainebleau, France, 2001). Variograms were plotted for each aggregate data set. The empirical variograms were subsequently modelled by fitting linear, spherical or exponential models.

Variograms describe the spatial correlation of variables as a function of inter-sample separation distance. In general, two neighbouring samples are more likely to have similar properties than 2 samples further apart. Therefore, inter-sample variance is lower between neighbouring samples than between samples separated by greater distances. This is reflected in the shape of semivariograms, where semivariance values increase as a function of inter-sample distance until a plateau (sill) is reached, after which there are no further trends with inter-sample separation distance. Three parameters can be obtained from semivariograms; these are discussed below.

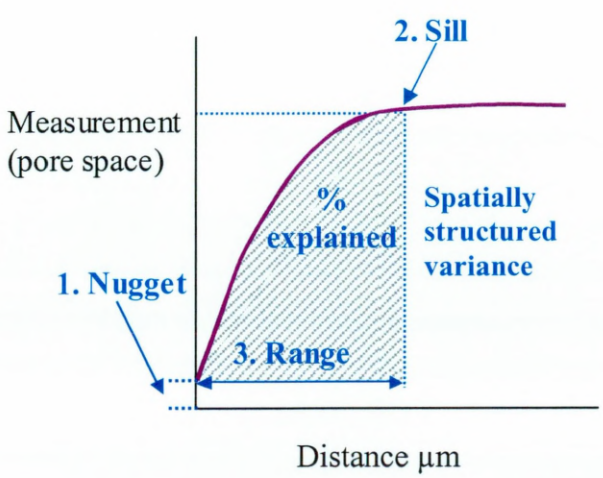


Fig. 4.5 Associated values extrapolated when using variogram analysis, the shaded area highlights the percentage of variance explained.

1. **Nugget variance** is the discontinuity at the origin, suggesting that a sample is different from itself. This inconsistency is generally attributed to experimental error (here, possibly errors in micro-tomographic reconstruction and image analysis) and to variation at scales below the minimum scale of measurement, in this case <4.4 μm.
2. **Sill**: the sill is the plateau in the empirical variogram, where inter-sample variance no longer depends on inter-sample distance. The sill represents the total sample variance.
3. **Range**, is the distance between observations beyond which there is no spatial correlation.

From the values above, the percentage of variation explained (spatial or structural variation) can be calculated by subtracting the nugget value from the sill and then division by the sill. To determine these parameters one of a number of models may be fitted to the empirical variograms (Fig. 4.6).

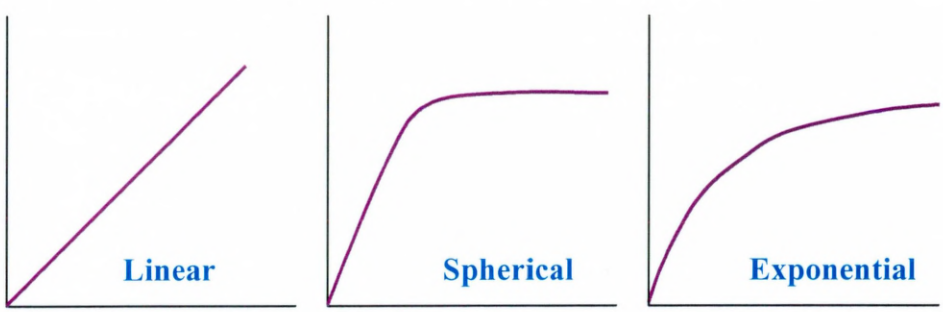


Fig. 4.6 Schematics of various variogram models, which can be applied to empirical variograms.

Omnidirectional variograms were not constructed because of computational constraints. Therefore, each of the aggregate variograms was plotted in three directions (x, y & z). Range, sill and nugget values were determined for each direction. Nugget values were expressed as a percentage of the total variance (i.e. sill values). No consistent anisotropy was observed and therefore the values determined were averaged. Differences in average parameter values among treatments were assessed using ANOVA (Genstat, Release 6.1).

4.3 Results

4.3.1 Pore analysis

Analysis was completed on only four (of five) image sets of the 30 day unplanted treatment as the images in this set were of very poor quality. These images could not be successfully cleaned and segmented using the macro that was suitable for all other images. Therefore, for consistency this image set was excluded from any further analysis.

Percentage porosity

There were no significant differences between zero day and unplanted after 30 days incubation treatments ($P>0.05$). Both of these had significantly lower levels of porosity than the other two treatments. Significant differences in porosity were detected between planted outer and planted inner aggregates ($P<0.05$). The greatest percentage porosity was detected in the planted inner treatment. The porosity values are shown in Fig. 4.7.

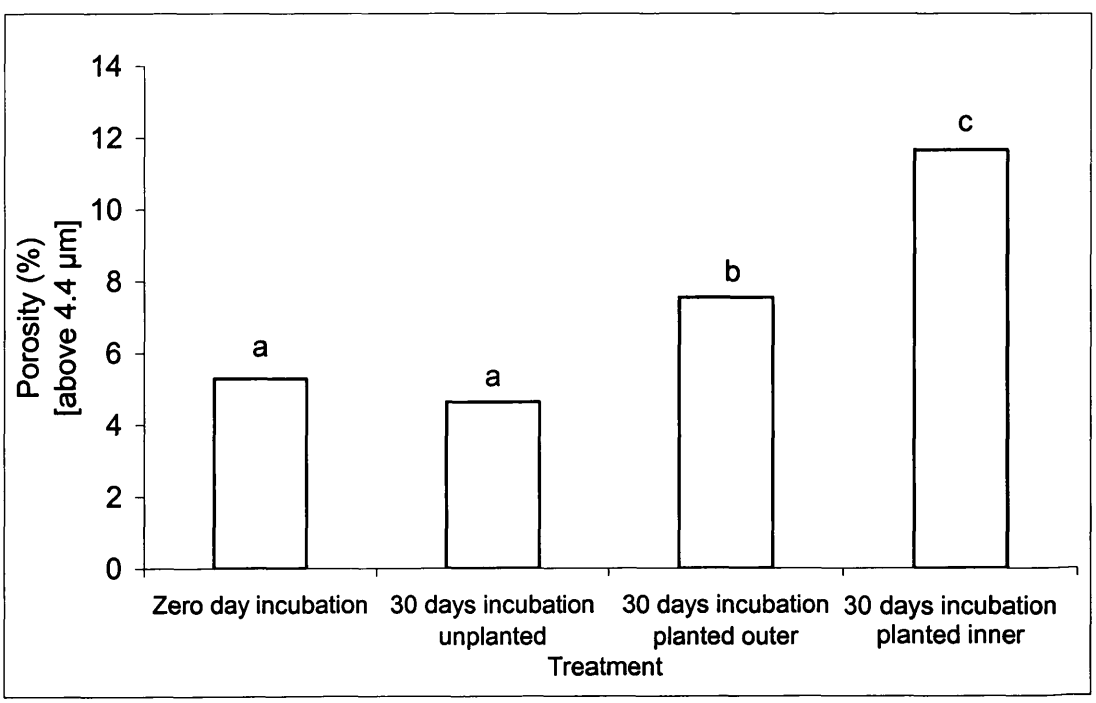


Fig. 4.7 Resulting percentage porosity established through image analysis of 3D CT scanned soil aggregates. Different letters represent significant differences ($P<0.05$).

Variogram analysis

Two exponential variogram models with a nugget provided a good fit for all the empirical variograms, and therefore were used to estimate the characteristics of the empirical variograms. The level of explained structural variance was very high (84-87%) and did not differ between treatments. The resulting values determined from modelled variograms are displayed in Table 4.1, whilst sample variograms for all treatments are shown in Fig. 4.9a-d.

No differences in nugget (expressed as percentage of total variance) between treatments were detected, suggesting that variation below the scale of measurement (<4.4 μm) and experimental error did not differ across treatments.

Aggregates from zero days incubation and 30 days incubation unplanted, were not statistically different in either range or sill (Table 4.1, Fig. 4.8a & b). There were however, significantly different spatial variances between other treatments. The largest average range and sill values were detected in (30 days incubation) planted inner aggregates, whilst the smallest range and sill values were detected in zero day (and unplanted) aggregates. Aggregates from the 30 days incubation planted outer treatment expressed average range and sill values somewhere between the two extremes. The significantly greater sill values detected in planted samples (both outer and inner) are indicative of greater variability.

Sample	Range	Sill (Transformed)*	Structural Variance (%)	Nugget (% total variance)
Zero day	222.1	0.20	86.44	13.56
30 days unplanted	204.2	0.21	84.42	12.58
30 days planted outer	259.9	0.24	86.84	13.16
30 days planted inner	320.6	0.29	87.59	12.41
LSD	28.86	0.026	N/A	N/A
P-Value	0.004	0.009	N/A	N/A

Table 4.2 Resulting range, sill, nugget (as percentage of total variance) and structural variance values from variogram analysis of pore spaces within 3D CT scanned soil aggregates. Associated least significant difference (LSD) and *P-values* are shown. *Sill values were not normally distributed and were square root transformed for analysis.

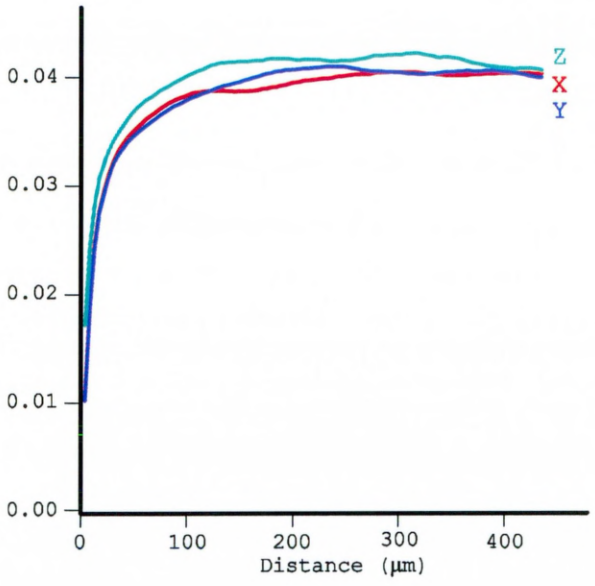


Fig. 4.8a Zero days incubation.

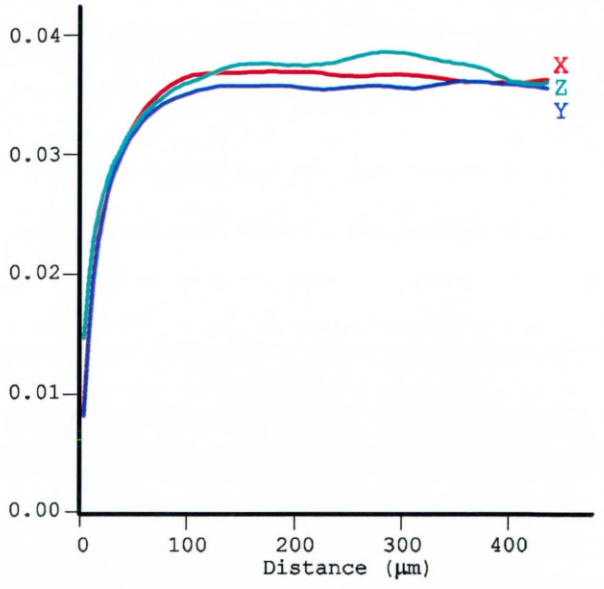


Fig. 4.8b 30 days unplanted.

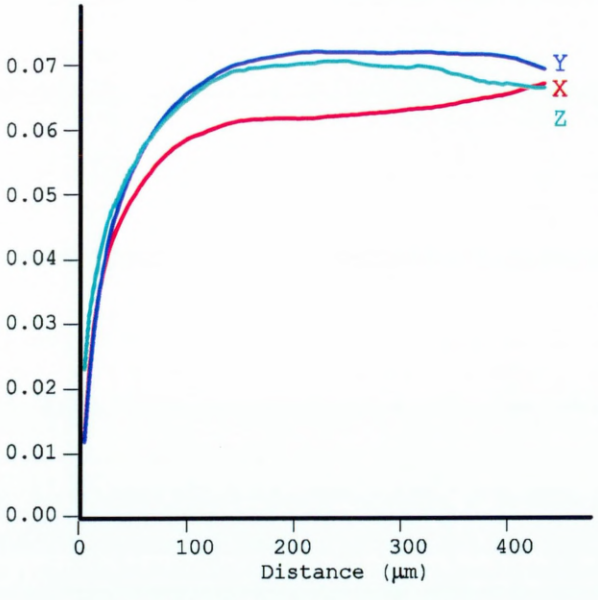


Fig. 4.8c 30 days planted outer.

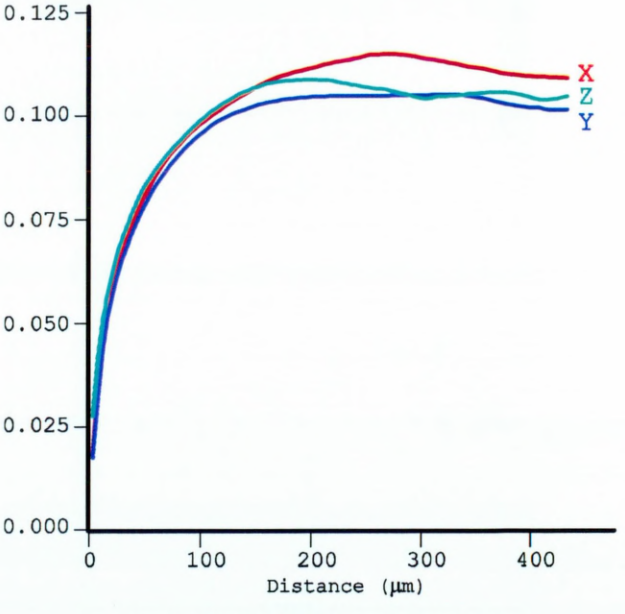


Fig. 4.8d 30 days planted inner.

Fig. 4.8a-d Empirical variograms of pore space within soil aggregates. Variograms were fitted with two exponential models. Each plot illustrates the three directional variograms.

Some 3D reconstructions of example images, with cutouts revealing internal porosity, completed using Slicer Dicer (Pixotec, Renton, WA, USA) are shown in Fig. 4.9a-d.

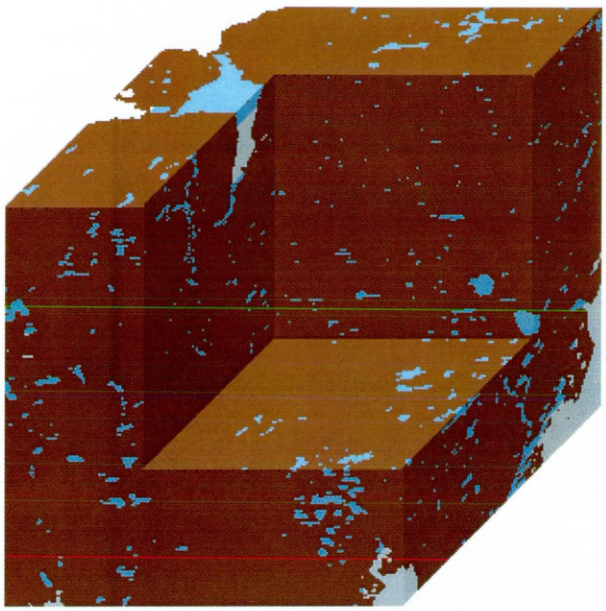


Fig 4.9a Zero days incubation.

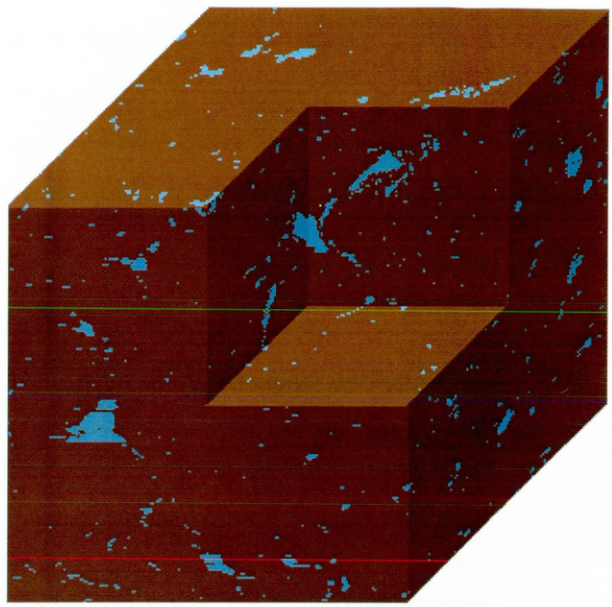


Fig. 4.9b 30 days incubation unplanted.

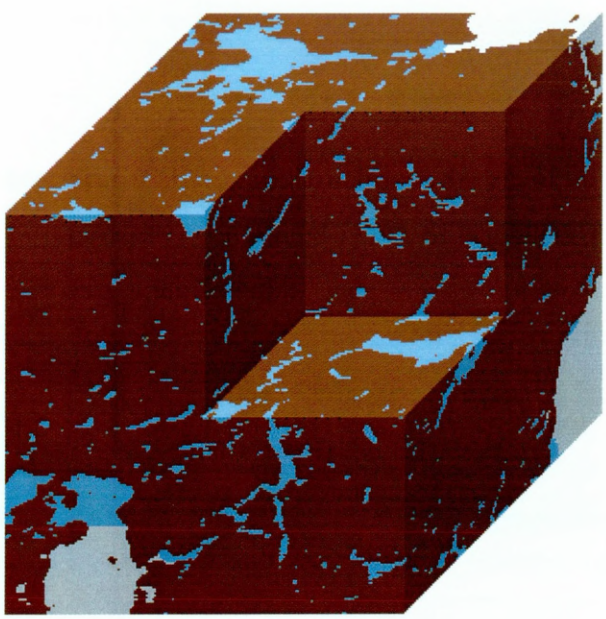


Fig. 4.9c 30 days incubation planted outer.

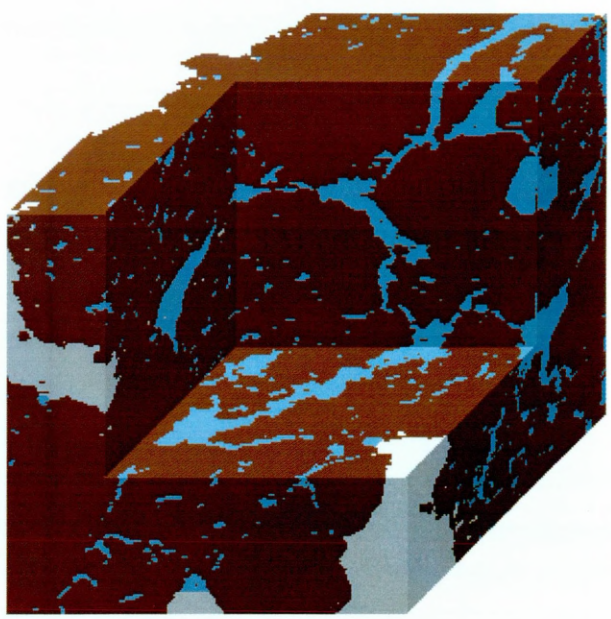


Fig. 4.9d 30 days incubation planted inner.

Fig. 4.9a-d 3D reconstructed images using Slicer Dicer representing the internal structure of soil aggregates, cubes are 200×200 pixels (equal to 892 × 892 μm). Solid matter is represented by brown whilst pore space is represented by blue.

4.3.2 Spatial analysis of solid matter

Variograms were plotted for "solid" image data sets to detect for spatial variation within the non-pore section of aggregate images, this being the predominantly mineral portion of the soil aggregate. A nugget effect and two further models were modelled against the empirical variograms. In all cases the initial model applied to variograms was an exponential, then further linear, exponential or spherical models were applied to variograms. The application of linear models to some of the data sets indicated that the range investigated in this case was not large enough to allow the detection of observations that were no longer autocorrelated. Additionally, the use of three different models makes further analysis difficult. No significant differences between nugget values were detected between any of the treatments ($P>0.05$). No further analysis was applied to the solid data sets. Two sample variograms are shown in Fig. 4.10, illustrating the typical spread of data for some of the linear modelled variograms.

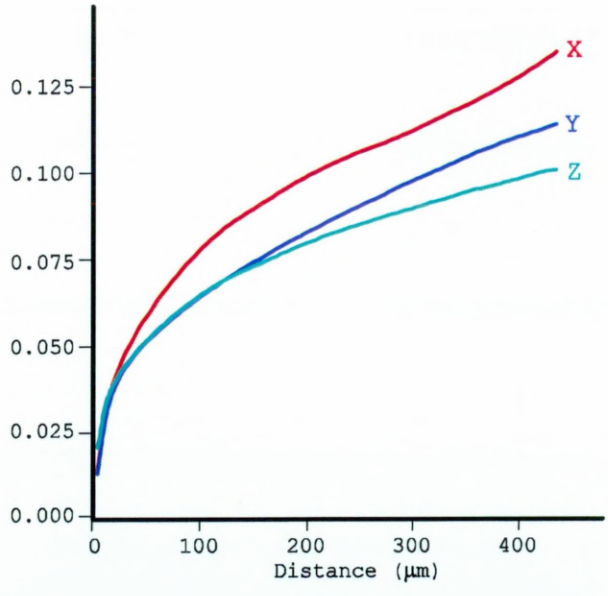


Fig. 4.10a Zero days incubation, variogram of solid data.

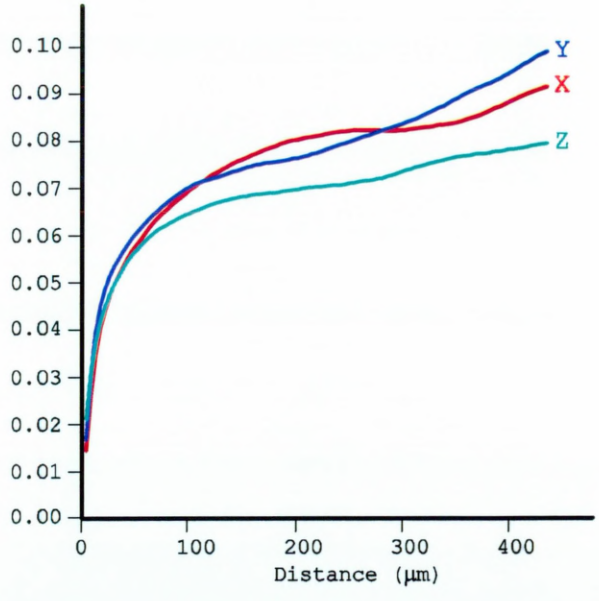


Fig. 4.10b 30 days planted inner, variogram of solid data.

Fig. 4.10a-b Empirical variograms of solid matter distribution within soil aggregates. Variograms were fitted with an exponential model, and a further linear, exponential or spherical model. Each plot illustrates the three directional variograms.

4.4 Discussion

The application of the experimental perturbations presented in Chapter 3 resulted in differing levels of porosity detected through the application of X-ray CT and image analysis of soil aggregates. Further to this was the detection of significant spatial differences in pore space between the treatment groups. The geostatistical analysis of soil aggregate pore space demonstrated that aggregates from unplanted and zero days incubation had small, evenly spaced pores, whilst planted outer and planted inner aggregates had increasingly larger pores, which were less evenly distributed within the aggregates (this is illustrated in the images shown in Fig. 4.9).

Investigation of the spatial distribution of solid matter did not result in the successful statistical analysis of image data sets. The use of linear models in some of the experimental variograms meant that no associated range value could be determined, indicating that the scale investigated, in this case, was too small (Houlding, 2000). Successful analysis of data of this nature would require the use of larger scale images to allow the detection of variance, as the scale investigated (200×200 pixels) was too small to detect changes. The analysis could have been completed on larger portions of images isolated from aggregates, for example cropping the images to 300×300 pixels. In some cases, however, this could have increased the impact of edge effects by including more aggregate edges and thus, not necessarily improving the analysis. Another option could be, in future to use larger soil aggregates, however this might result in lower resolution.

Increases in total porosity and changes in the spatial distribution of pore space were most pronounced in planted inner aggregates. Planted outer aggregates, however, also demonstrated a similar but less marked increase in pore space and spatial variation. On the basis of the results in Chapter 3, it was originally hypothesised that a first order effect on porosity would result from fungal mediation of structure. However, after 30 days incubation where the largest increase in porosity was detected no comparable differences in fungal biomass were found between planted inner, outer or unplanted soil treatments. This indicates that fungi may not be

directly responsible for changes, or that fungal biomass may not be an appropriate measure and quantifying fungal exudates may prove more useful.

Planted outer soil was linked to the rooted section (planted inner) and separated only by a 38 μm nylon mesh (see Fig. 3.1, Section 3.2.1). Therefore effects from root-mediated processes in this section may have been more pronounced than initially thought. Plants can cause localised drying, removing water from soil (Allison, 1968; Guidi *et al.*, 1985). Soil cores in this experimental set-up were watered to weight regularly so the soil may have been subject to slight wet~dry cycles. Unplanted cores would have been subjected to the loss of water though evaporation (although this was minimised in the experimental set-up). However, planted cores would have lost additional water through evapotranspiration (Brady, 1974). This would have undoubtedly increased the magnitude of wetting and drying in planted samples. Shrinking and swelling processes are capable of changing the physical properties of a soil (Semmel *et al.*, 1990). Increases in porosity may have been induced as a result of soil wetting and drying, a phenomenon reported also by Czarnes *et al.* (2000). The effect of wet~dry cycles on soil can also increase the stability of soil aggregates, with Denef *et al.* (2001) demonstrating that after three wet~dry cycles aggregates >2000 μm became resistant to slaking. In addition to the physical enmeshment of soil particles by roots, the influence of wetting and drying of soil may also partially explain the increase in aggregates >2000 μm reported in Chapter 3.

Assessing porosity in bulk soil and soil taken directly from plant roots Semmel *et al.* (1990) reported that porosity was greater in bulk soil in contrast to "rooted" soil. The methodology applied did not permit the visualisation of pore space, and the experiment used the root system of maize. The different plant type may have induced different changes in the root zone with maize well documented in its ability to exude extra-cellular mucilage and change soil properties (Watt *et al.*, 1994; Watt *et al.*, 1993). Guidi *et al.* (1985) stated that the wetting and drying of a homogenous soil could result in the "heterogenisation" of pores. This was visible in the planted aggregates presented in this chapter, where pores became larger and less evenly spaced. This can occur when a soil is wet. Particles can be rearranged under "*menisci forces and hydraulic gradient*" (Semmel *et al.*, 1990). This mechanism

along with the penetration of plant roots into available pore space will result in porosity size and physical distribution differences.

No differences were reported in water repellency measurements between the unplanted, planted outer and planted inner (30 days incubation) [Chapter 3, Section 3.3.4, Fig. 3.6b]. Increases in porosity across these treatments seems counterintuitive; with a greater volume of fillable pore space (i.e. greater porosity) it would be assumed that the wettability and sorptivity of the soil would increase, subsequently inducing a reduction in repellency levels. Czarnes *et al.* (2000) reported increases in porosity as a result of wet~dry cycles, and paired with decreases in repellency. In the case of the results presented here it is possible that the pore space was not directly accessible to infiltrating liquid, with hydrophobic substances having a direct impact upon repellency levels, with increased porosity detected in planted inner aggregates and not changes in water repellency.

4.5 Conclusions

Using X-ray CT for the first time, considerable differences were detected in total porosity and pore space distribution after only thirty days incubation, using only five replicate aggregates per treatments. The differences in porosity were likely to be as a result of a combination of localised wetting and drying, root and microbial activity. If changes in pore distribution and porosity were directly associated with root mediated effects, the detection of changes in the planted outer aggregates indicates that the zone of influence spans a substantial distance from the roots considering the scale of aggregates used in this investigation were ≈2 mm. Further work of this nature, would allow a greater understanding of how micro-scale changes in soils result from plant and microbial activity.

The methodology applied in this chapter allows the linkage of structural variations with changes in water infiltration, and aggregate stability in soil. Increasing the scale of the aggregates scanned and repeating the experiment, scanning samples after shorter time periods of incubation would give a more detailed indication of when

specific changes in pore distribution occur. More sophisticated analysis on pore connectivity and tortuosity, paired with other measurements, would give a greater insight into biological and physical soil processes.

Chapter 5: The impact of prolonged land management upon soil properties

5.1 Introduction

The application of nutritional amendments to agricultural soils allows for enhanced crop production, maintaining a soil's productivity without becoming nutrient deficient. The application of NPK fertilizers (nitrogen N, phosphate P_2O_5 and potassium oxide, K_2O), increases plant growth and activity (Russell, 1988), and undoubtedly has significant impacts upon soil microbiological populations and soil structural stability (Bossuyt *et al.*, 2001; Preston *et al.*, 1999). The application of N to soils directly affects soil acidification and root development. Sparse root development occurs in N deficient systems (Bethlenfalvay *et al.*, 1999; Donnison *et al.*, 2000), reducing the impact of roots in soil structural mechanisms and increasing the importance of AM fungi in the formation of water stable aggregates (Bethlenfalvay *et al.*, 1999).

In addition to direct and indirect affects upon microbial populations changes in soil pH as a result of fertilizer application may have an impact upon the stability of soil particles. Decreases in soil pH can result in reduced water stability of soil aggregates, due to interference between the binding of clays and organic materials by polyvalent cations (Oades, 1984). Additionally pH causes changes to fungal population dynamics with some isolates of AM fungi showing pH preferences (Abbott and Robson, 1991). Generally, total fungal biomass will remain constant at various levels of pH (Brady, 1974), however, low pH can induce reductions in bacteria and actinomycetes (Brady, 1974).

Roberson *et al.* (1995) reported that N application affected the slaking resistance of soil and levels of microbial extracellular polysaccharide, reporting a correlation between the two measurements. They also reported that the correlation between slaking resistance and microbial biomass was poor, noting that fungal biomass in particular was more likely to play a role in reduced disturbance systems rather than conventionally tilled systems. Fungal associations particularly mycorrhizal in arable soils are directly affected by tillage, the application of NPK fertilizers will either indirectly or directly influence fungal activity. Plants are more likely to form AM associations in phosphate poor soils (Joner, 2000; Russell, 1988), and conversely

less likely in phosphate rich environments. Additionally, the growth of different fungal species can either be inhibited or stimulated through the application of inorganic fertilizers (Donnison *et al.*, 2000). Given the importance of fungi in maintaining soil structure (Bearden and Petersen, 2000; Miller and Jastrow, 1990, 1992, 2000; Monlope *et al.*, 1987; Tisdall *et al.*, 1997) changes of this nature are important and require investigation in order to understand the indirect implications of fertilizer application.

Hallett and Young (1999) demonstrated that nutrient additions significantly increased levels of water repellency with corresponding increases in total microbial activity. This investigation used agricultural soils and offered no comparison with undisturbed grassland soils. A further investigation of this nature with measures of fungal biomass would provide an interesting assessment of nutritional impacts upon soil water processes and associated fungal influences.

The species specificity of some fertilizers upon fungi, and the fact that much of the research in this area has been on cultured organisms, whilst much of the fungal population particularly in grassland systems are made up of non-culturable organisms mean that the interactions between microorganisms, nutritional amendments and soil structural stability in the field is relatively poorly understood.

Nutritional input directly affects plant growth, and will both directly and indirectly affect microbial activity. These changes may subsequently change the structural properties of soil. The aim of this chapter is to investigate the long-term effects of differing fertilizer regimes upon various soils, comparing a long-term traditional arable field site with a long-term grassland and an organically managed field site. Investigating glomalin concentration in an undisturbed (grassland) soil, will allow further understanding of the relationship between repellency and glomalin, with the detection of glomalin at higher concentrations. In Chapter 3, a relationship between fungal biomass and water repellency was reported. This is examined further in this chapter by also considering the influence of fertilizer treatments upon fungal biomass and the resulting levels of subcritical repellency.

5.2 Materials and Methods

5.2.1 Information on field sites selected

5.2.1.1 Long-term grassland site: Palace Leas field site

Palace Leas field site is situated at Cockle Park Farm (The University of Newcastle) and has been established and not ploughed since 1896. Palace Leas treatments consist of a range of fertilizer treatments that upon establishment of the field site were considered best practice. Nutritional treatments consist of varying amounts of nitrogen, phosphorus, potassium and farmyard manure. The field site consists of 14 parallelogram shaped plots beside each other. Of the 14 plots, seven plots were sampled, selected on the basis that they would give a representative view of changes in fungal biomass and soil structure across the extremes of treatments. The plots and treatments applied are shown in Table 5.1

		Fertilizer (kg ha^{-1})		
Plot	Farm Yard Manure (t ha^{-1})	N	P_2O_5	K_2O
1	20	17	30	34
2	20			
6				
7		35		
10		35	60	
11		35		37
14*		100	66	100

Table 5.1 Palace Leas treatments applied annually to sampled plots. Information taken from Shiel, (2000a). *Plot 14 was established in 1976.

5.2.1.2 Long-term arable site: Broadbalk field site

The experimental field site of Broadbalk situated at Rothamsted Experimental Station (Harpenden, UK) was selected as the long-term arable. Plots were established in 1843 and were originally designed to test the impact of inorganic supplements upon crop yield, and the effects of continuously cropping the same crop (Institute of Arable Crops Research, AFRC, 1991). The field site is divided into sections, which contain different crop treatments consisting mainly of continuous wheat and further sections housing different cropping rotations. Cropping sections are subdivided into a series of strips, which separate the various fertilizer and organic manure treatments. From the arable field plots, six samples were taken; continuous wheat (Section 9, 3 × fertilizer treatments) and continuous wheat restricted pesticide application (Section 6, 3 × fertilizer treatments). The restricted pesticide treatments had no summer/spring fungicides applied. Included in this field site were three additional non-cropped plots labelled "Wilderness", established in 1882. This 0.2 ha area was fenced off from the arable field plot, and is split into a woodland area, established grassland and a stubbed section that allows open ground vegetation to develop (Institute of Arable Crops Research, AFRC, 1991). Two samples were taken from the wilderness section, one from each of the grazed and wooded areas.

The three fertilizer treatments selected were, Strip 3: no fertilizer application, Strip 8: N-144 kg ha^{-1}, P-35 kg ha^{-1}, K-90 kg ha^{-1}, Mg-30 kg ha^{-1}, and Strip 2.2: FYM-35 t ha^{-1}, with fertilizer amounts indicating the amount applied annually. Samples taken were selected to give a reasonable range of land management or fertilizer treatments. Soil from eight of the experimental plots were sampled.

5.2.1.3 Stockless arable organic soil: Terrington experimental field site

Soil was sampled from a field scale study run by the Agricultural Development and Advisory Service (ADAS) at Terrington (Norfolk, UK). The site has been developed as a long-term field site to study the sustainability of stockless arable organic production. The field site is a 10 ha area split into a number of fields with a five-year crop rotation in place. Organic farming commenced in 1990 and was phased

into the different plots so that each field is at a different stage of rotation. Four ADAS Terrington plots were sampled, two that are currently conventionally farmed, and two that have been organically farmed for the past 10 years; the rotation stage of each plot along with fertilizer treatments are shown in Table 5.2.

Field	*Management*	*Fertilizer treatment (2003)*	*Rotation 2000*	*Rotation 2001*	*Rotation 2002*	*Rotation 2003*
1	Organic	Reddzlagg * (625 kg ha^{-1})	Potatoes	Winter wheat	Spring beans	S. Barley
2	Organic	No fertilizer	Clover	Potatoes	Winter wheat	Spring beans
3	Conventional	N: 190 kg ha^{-1}	Winter wheat	Winter wheat	W. OSR	Winter wheat
4	Conventional	N: 290 kg ha^{-1} P: 300 kg ha^{-1}	Sugar beet	Winter wheat	Winter wheat	W. OSR*

Table 5.2 Terrington conventional and stockless organically farmed plots, details of crop rotations and fertilizer treatments. *Reddzlagg is an ammonium citrate soluble phosphate, approved by the Soil Association. (*OSR=oil seed rape)

5.2.1.4 Supportive experimental data

Data produced by the various field sites detailing the chemistry of the individual plots is presented in Table 5.3. Fertilizer treatments applied to Palace Leas and Broadbalk soils have induced slight changes in pH and considerable changes to soil nutrient levels in particular soluble phosphate, which may affect mycorrhizal associations. The application of FYM to agricultural soil increased the percentage soil C and N to levels equivalent of the undisturbed systems.

		Mean Values for 2003							
Field site	Plot/Treatments	K (per kg^{-1})	P $mg\ kg^{-1}$	Mg (per kg^{-1})	Ca per kg^{-1}	pH	N %	Org C %	Hay yield $t\ ha^{-1}$
Palace Leas	1	6.91 m mol_c	134	26.8 m mol_c	125 m mol_c	5.5			6496
Palace Leas	2	5.10 m mol_c	126	14.1 m mol_c	114 m mol_c	5.4			5920
Palace Leas	6	4.01 m mol_c	3	13.6 m mol_c	45.5 m mol_c	5			2554
Palace Leas	7	4.13 m mol_c	0	6.9 m mol_c	36.5 m mol_c	3.8			3049
Palace Leas	10	4.31 m mol_c	32	13.6 m mol_c	75.8 m mol_c	4.7			4113
Palace Leas	11	6.72 m mol_c	0	6.2 m mol_c	22 m mol_c	3.7			2923
Broadbalk	Wheat, no fertilizer	81 mg	7	34 mg	4960 mg	8.0	0.084	0.83	
Broadbalk	Wheat + N, P, K, Mg	274 mg	78	67 mg	2640 mg	7.0	0.117	1.23	
Broadbalk	Wheat + FYM	684 mg	102	118 mg	4460 mg	7.6	0.288	3.02	
Broadbalk	RP, Wheat, no fertilizer	113 mg	8	34 mg	5500 mg	8.1	0.089	0.84	
Broadbalk	RP, Wheat + N, P, K, Mg	309 mg	83	75 mg	2720 mg	7.1	0.109	1.09	
Broadbalk	RP, Wheat + FYM	693 mg	90	121 mg	4600 mg	7.6	0.266	2.81	
Broadbalk	Wooded	260 mg	260	172 mg	6700 mg	7.7	0.290	3.39	
Broadbalk	Grazed	185 mg	185	100 mg	5780 mg	7.4	0.286	3.05	
Terrington	1 Organic						0.20	2.65	
Terrington	2 Organic						0.27	3.36	
Terrington	3 Conventional						0.17	2.64	
Terrington	4 Conventional						0.23	3.25	

Table 5.3 Supportive data on experimental field sites. Where no information was available space remains blank. Palace Leas data taken from Shiel (2000b) no data was available for Palace leas plot 14. Broadbalk data from personal communication, Paul Poulton (2003).

5.2.2 Sampling

The Palace Leas field site was sampled in August 2004; soil was randomly sampled within each treatment at 50-70 mm depth in order to avoid the thick thatch layer. Five replicate samples were taken across each plot, bulked and later homogenised allowing one representative sample per plot, avoiding any spatial variation or influences.

Broadbalk filed site was sampled in August 2003, soil was taken between 20-50 mm depth; samples were taken in five areas from each treatment and bulked together to give an even representation minus the impact of any spatial bias.

The ADAS Terrington field site was sampled in July 2003, soil was taken from three different locations across each field; soil was taken from the top 20-50 mm.

Prior to further analysis all soils were stored at 4°C for no longer than a period of two days.

5.2.3 Glomalin and ergosterol isolation

Prior to extraction all soils were sieved to 2 mm to homogenise and remove large stones. Five ergosterol and five glomalin extractions were made per plot. Ergosterol and glomalin were extracted as detailed in Sections 3.2.3 and 2.2.3 respectively. Ergosterol and glomalin isolation was completed on soil samples within two days of sampling.

5.2.4 Water repellency

The methodology described in Sections 1.3.4.4 and 2.2.4 was used to assess water and ethanol sorptivity to establish water repellency. Two differing measures of water repellency were applied to sampled soils. Measurements were completed on intact aggregates taken directly from the sampled soil. Aggregates were approximately 3-5 mm in diameter and selected by avoiding those with particularly large root segments and stones. The second repellency measurement was carried out upon the surface of packed soil cores. Small, 10 mm in height, 20 mm diameter plastic cores were packed with soil sieved to >2 mm at 1.3 Mg m^{-3} dry bulk density. Terrington soil samples were partially sieved prior to analysis with no, or very few intact aggregates within the samples; therefore only one measure of water repellency was made upon this soil.

All soils had five replicate measurements per plot and repellency was measured as detailed in Section 2.2.4.

5.2.5 Statistical analysis

The resulting water repellency, glomalin and ergosterol levels were compared using an ANOVA, whilst comparisons between the measurements were made using a Pearson correlation (GenStat, Release 6.1).

5.3 Results

Due to the very different nutritional treatments applied to each of the field sites, results of the different soils will be presented separately.

5.3.1 Long-term grassland: Palace Leas field site

5.3.1.1 Impact of fertilizer treatments upon fungal biomass

Analysis of ergosterol to indicate fungal biomass resulted in the detection of considerable concentrations of ergosterol and significant effects of nutrient additions as illustrated in Fig. 5.1, ranging from a maximum average of 35.9 μg g^{-1} in plot 7 which received nitrogen only, to a minimum average of 2.7 μg g^{-1} in plot 1, which received farm yard manure, N, P & K, all differences significant to $P<0.01$. The application of P appeared to reduce ergosterol concentrations (as did the application of FYM) with Plot 6, which received no nutritional amendments, expressing significantly greater concentrations in comparison.

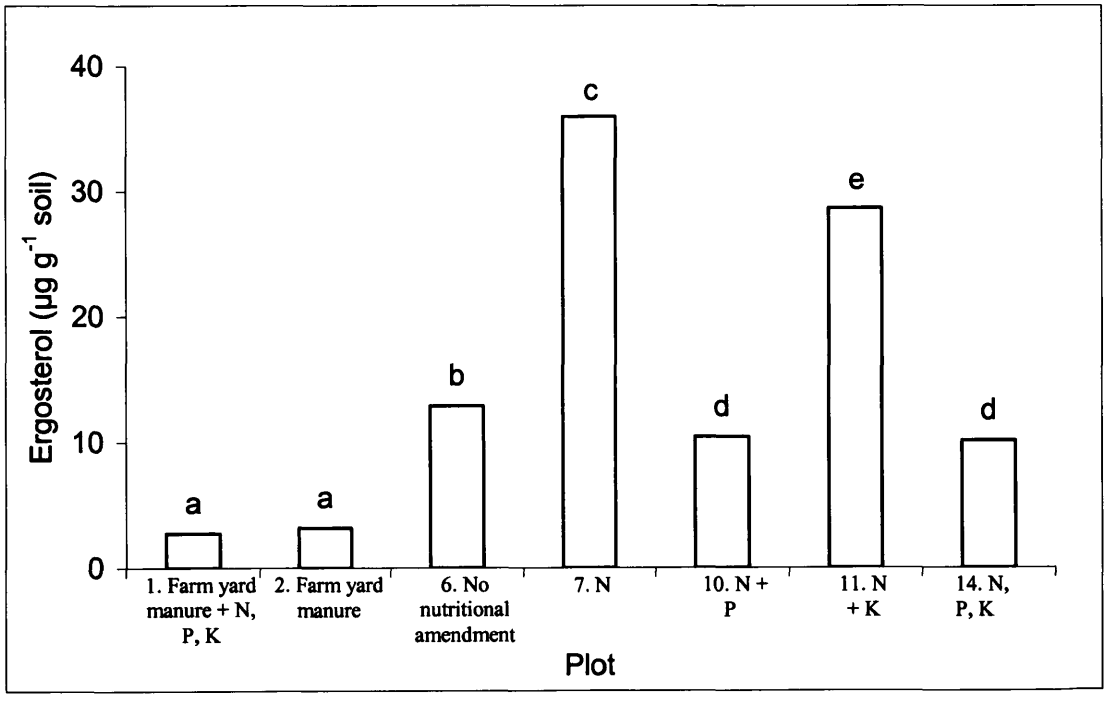


Fig. 5.1 Ergosterol concentrations from the long-term grassland site, Palace Leas. Different letters denote significant differences ($P<0.01$) between plots.

5.3.1.2 Impact of fertilizer treatments upon glomalin concentration

Glomalin concentrations detected in soil from Palace Leas long-term grassland plots are shown in Fig. 5.2. Glomalin concentration, similar to ergosterol, was detected at considerable concentrations and with significant effects of nutrient addition. The highest average concentration of glomalin at 18.7 mg g^{-1} was detected under Plot 7 where only nitrogen was applied to the soil. Whilst the lowest average concentration of 2.8 mg g^{-1} was detected under plot 6, which received no nutritional additions, Plot 10 (N+P additions) also expressed reasonably low concentrations of glomalin at 3.77 mg g^{-1}.

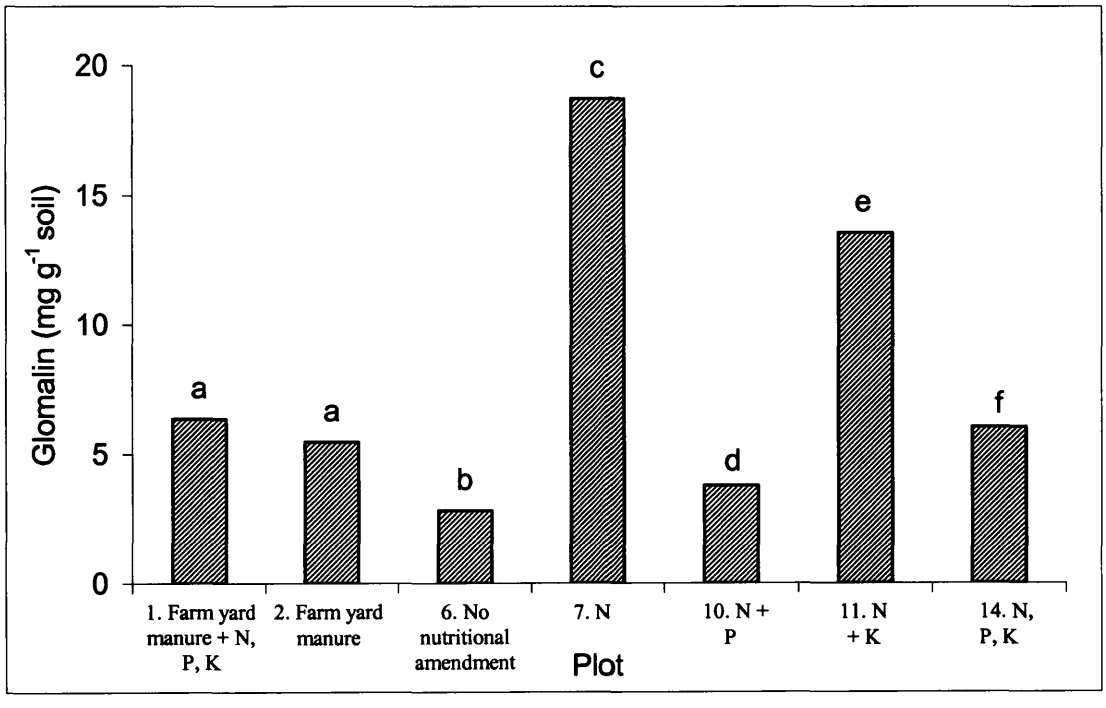


Fig. 5.2 Glomalin concentrations from the long-term grassland site, Palace Leas. Different letters denote significant differences (P<0.01) between plots.

5.3.1.3 Impact of fertilizer treatments upon water repellency

The resulting impact of nutritional amendments upon water repellency (illustrated in Fig. 5.3) was significant in both intact aggregates and re-packed cores, although the differences between the two measures varied.

Repellency of soil aggregates was more variable, indicated by an increased *c.v.* (coefficient of variation) in contrast to core measurements. Plot 11 expressed the greatest levels of repellency at 43.1, which was significantly different from all other plots. This was followed by plot 7, but the increased variation in this measure meant that this was statistically similar to plots 1, 2, 6 and 14.

Analysis of repellency in soil cores gave slightly different results, again no differences were detected between plots 1, 2, 6 and 14. However, in this case the highest repellency levels were detected in Plot 7 at 30.9 in a similar pattern to ergosterol and glomalin analysis. Plots 10 and 11 were statistically similar showing lower levels of repellency than Plot 7 but significantly greater levels than the other plots.

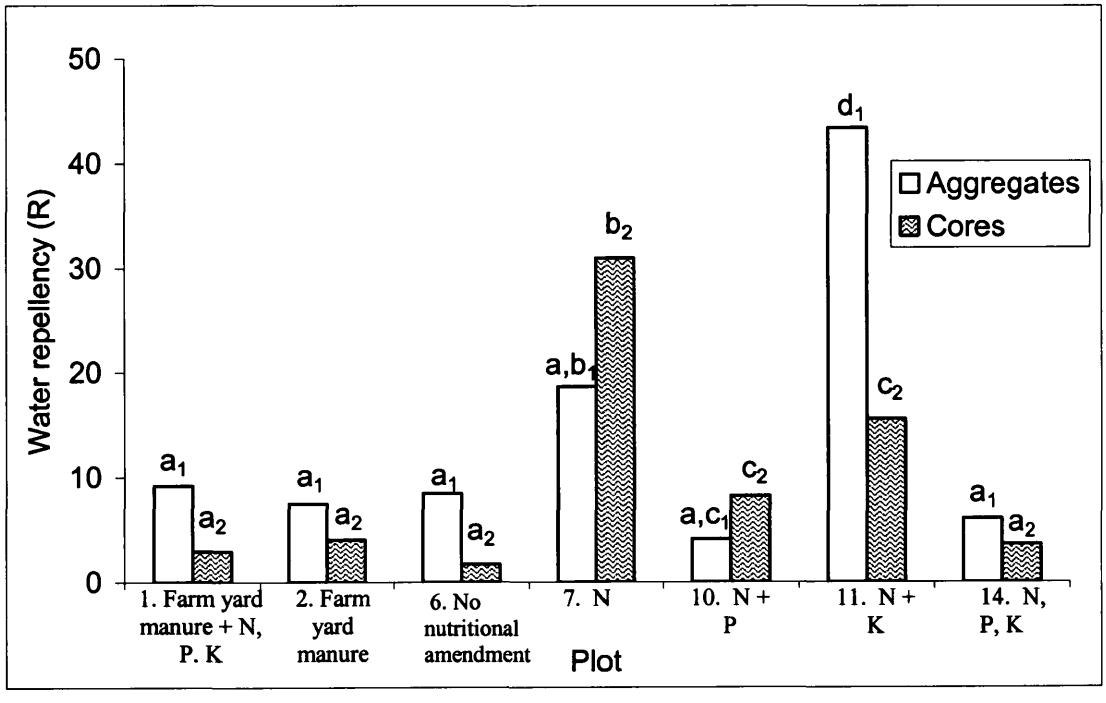


Fig. 5.3 Long-term grassland site, Palace Leas, water repellency levels upon intact aggregates and re-packed soil cores. Different letters denote significant differences ($P<0.01$) between plots.

5.3.2 Long-term arable soil: Broadbalk field site

5.3.2.1 Impact of fertilizer treatments upon fungal biomass

Levels of ergosterol detected in soils from Broadbalk long-term arable plots are shown in Fig. 5.4. Concentrations of ergosterol ranged from 0.8 μg g^{-1} for both wheat no fertilizer and restricted pesticide (RP): wheat no fertilizer, increasing to 1.6 μg g^{-1} under RP: Wheat + FYM. Wilderness plots expressed significantly greater (P<0.01) concentrations of ergosterol at 4.2 and 4.4 μg g^{-1} for wooded and grazed areas respectively.

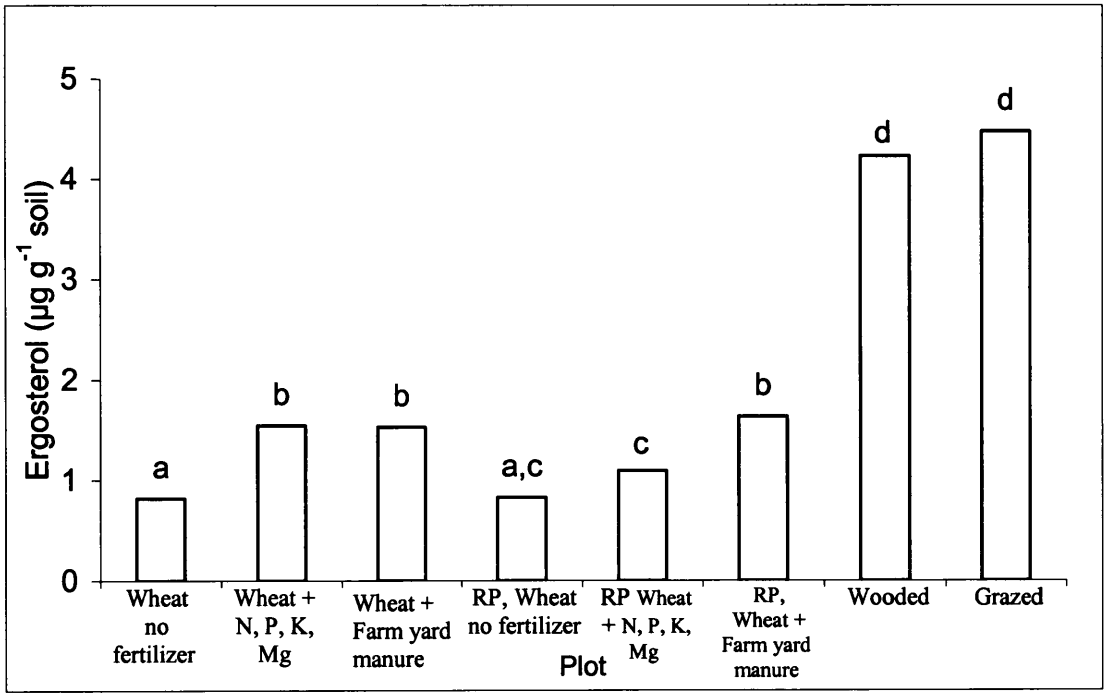


Fig. 5.4 Ergosterol concentrations from the long-term arable site, Broadbalk. RP indicates restricted pesticide treatments. Different letters denote significant differences (P< 0.01) between plots.

5.3.2.2 Impact of fertilizer treatments upon glomalin concentration

Very low concentrations of glomalin (0.3-0.6 mg g^{-1}) were detected under most continuous wheat plots including restricted pesticide plots. However, even at these low concentrations significant differences between plots were found (P<0.01) (Fig.

5.5). The exception to these low glomalin levels were wheat plots supplemented with FYM, which had concentrations of 2.3-2.5 mg g^{-1}. These levels were significantly greater compared to the other four wheat plots (P<0.01) and statistically similar to the wooded section which had an average concentration of 2.3 mg g^{-1} (P>0.01). The grazed section had significantly lower levels of glomalin 1.7 mg g^{-1} compared to plots supplemented with FYM and the wooded section, but greater than unfertilized and fertilized wheat plots (P<0.01).

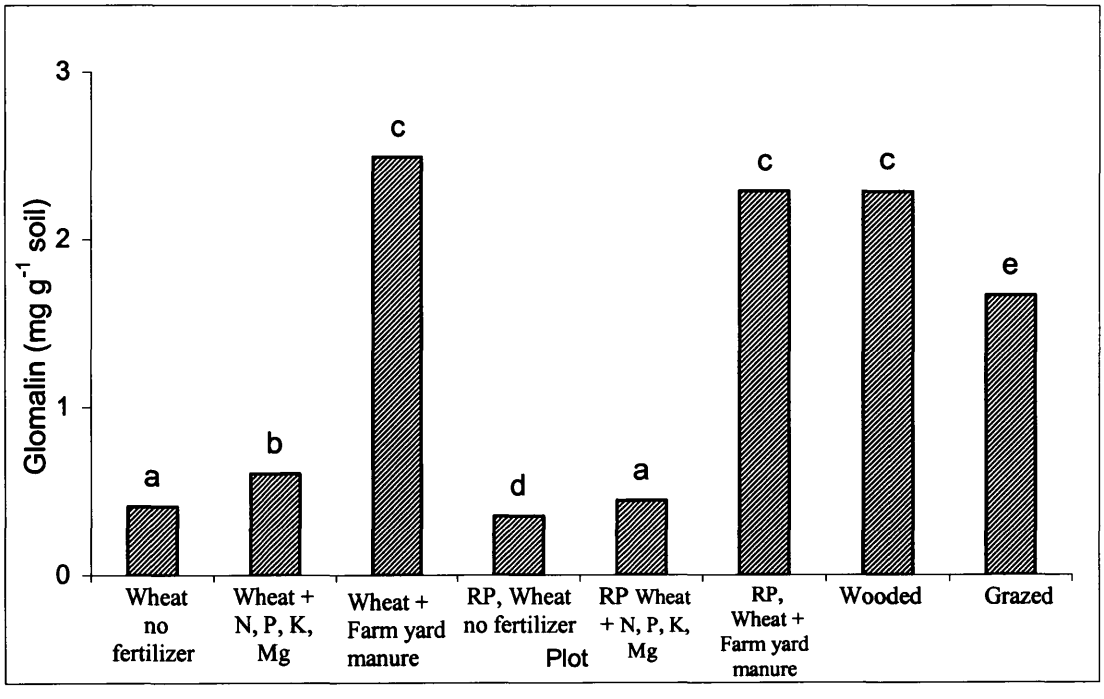


Fig. 5.5 Glomalin concentrations expressed in the long-term arable site, Broadbalk. RP indicates restricted pesticide treatments. Different letters denote significant differences (P< 0.01) between plots.

5.3.2.3 Resulting impact of fertilizer treatments upon water repellency

Water repellency results for the long-term arable field site of Broadbalk from both soil aggregates and soil cores are shown in Fig. 5.6. Levels of repellency measured in soil aggregates extracted from the field did not show significant differences between different plots (P>0.01), with the exception of the grazed "wilderness" plot, which had a significantly greater level of water repellency, than all other plots (P<0.01).

Measuring water repellency in re-packed soil cores resulted in the detection of increased differences between treatments. Lowest levels of repellency were detected in the four wheat plots that had either no fertilizer treatments applied or were amended with N, P & K. The two wilderness plots (wooded and grazed) along with the FYM restricted pesticide wheat plot all had significantly greater (P<0.01) levels of repellency than the other wheat plots, with an average of the three levels being between 1.9-2.1. There were no significant differences between these three plots. The continuous wheat amended with FYM had a statistically similar level of repellency to these three plots but was additionally statistically similar to the other wheat plots (See Fig. 5.6 for more details).

In contrast to the previous soils, variation between the two measurements of water repellency was minimal with the *cv.* (coefficient of variation) similar for both aggregate and core measures.

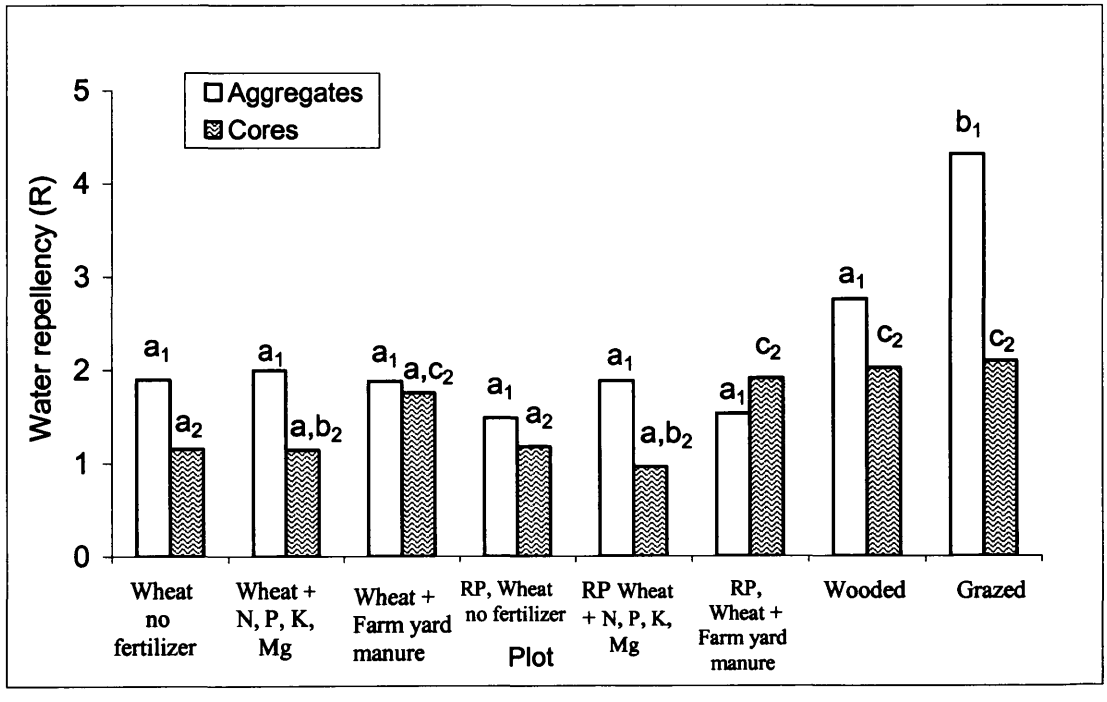


Fig. 5.6 Long-term arable site, Broadbalk, water repellency levels upon intact aggregates and re-packed soil cores. RP indicates restricted pesticide treatments. Different letters denote significant differences (P< 0.01) between plots.

5.3.3 Stockless arable organic soil: Terrington

5.3.3.1 Impact of arable organic practices upon fungal biomass

Ergosterol concentrations detected in the Terrington field site are shown in Fig. 5.7. No significant differences between the three different locations sampled within each of the fields were detected ($P>0.01$). The lowest concentrations of ergosterol detected were in Field 3, a conventionally managed field; the concentrations in this field were significantly lower than all other fields ($P<0.01$). The highest concentrations of ergosterol were detected in an organically managed field (Field 2), which averaged 3-3.5 μg g^{-1} of ergosterol. There were no significant differences between Field 1 (organic) and Field 4 (conventional) [$P>0.01$].

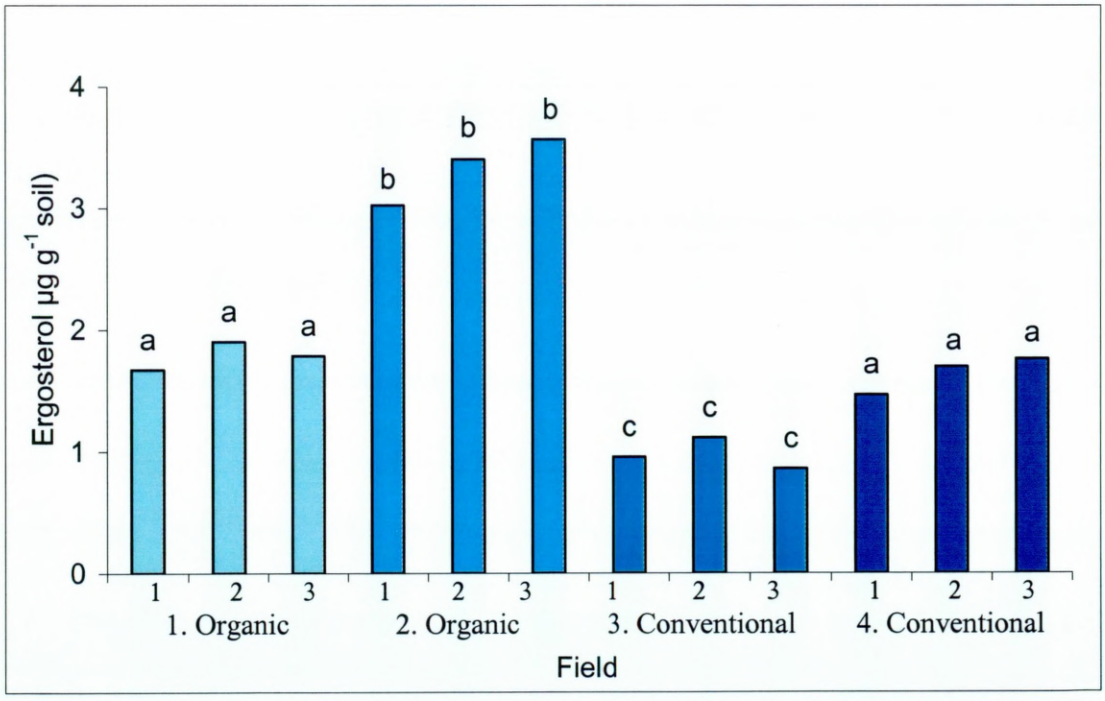


Fig 5.7 Ergosterol concentrations expressed in the stockless arable organic soil, Terrington, four fields were sampled with three different locations per field represented as 1-3. Different letters denote significant differences ($P< 0.01$) between plots.

5.3.3.2 Impact of arable organic practices upon glomalin concentration

There was little spatial variation in glomalin concentrations within fields ($P>0.01$), with the exception of Field 3 where sample 1 differed from samples 2 & 3 ($P<0.01$). The highest concentration of glomalin was in Field 4, a conventionally managed field where glomalin was in the range of 1.8-2 mg g^{-1}; these values were significantly greater than all other fields ($P<0.01$). The remaining three fields had similar levels of glomalin with various differences between plots, which are illustrated in Fig. 5.8.

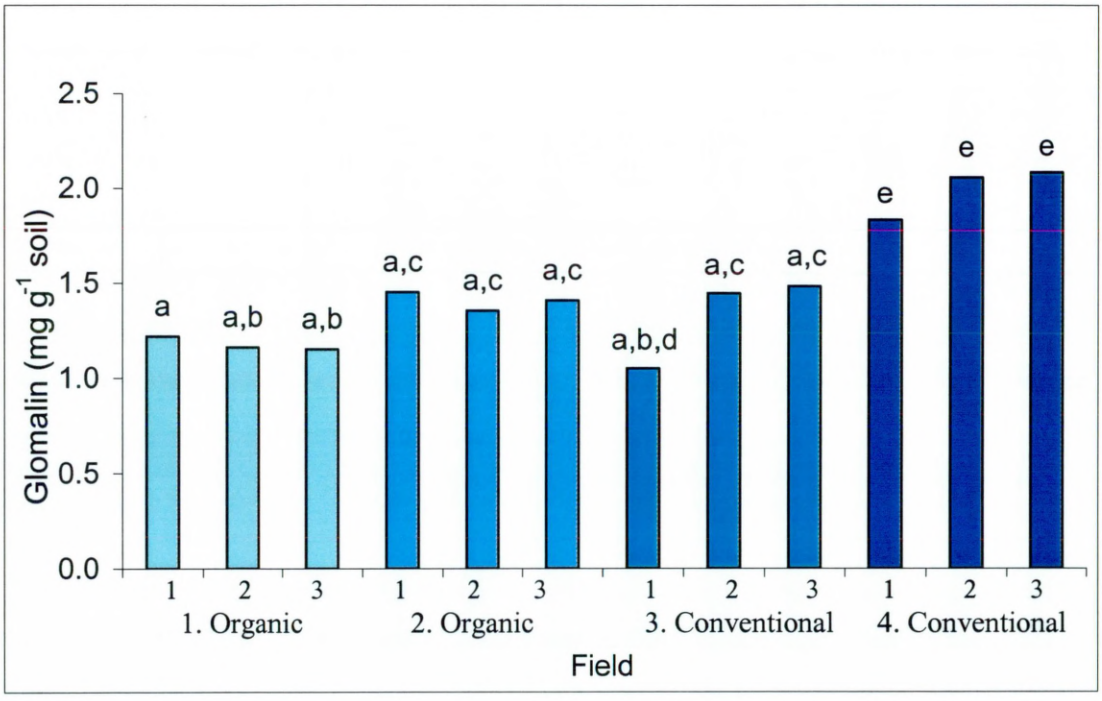


Fig 5.8 Glomalin concentrations expressed in the stockless arable organic soil, Terrington, four fields were sampled with three different locations per field represented as 1-3. Different letters denote significant differences ($P< 0.01$) between plots.

5.3.3.3 Impact of arable organic practices upon water repellency

Unlike the previous two field sites, Terrington soils were not subjected to two measures of water repellency. The soils were partially sieved prior to analysis and as a result no intact aggregates were available for aggregate measures of water

repellency. Thus the analysis of water repellency for this soil was assessed upon re-packed soil cores only, the results of which are shown in Fig. 5.9.

Similarly to measures of ergosterol and glomalin, there was no significant spatial variation within fields in water repellency levels (P>0.01). However, significant differences in water repellency levels between fields were detected only between various combinations of spatially located samples, and were not detected between fields as a whole.

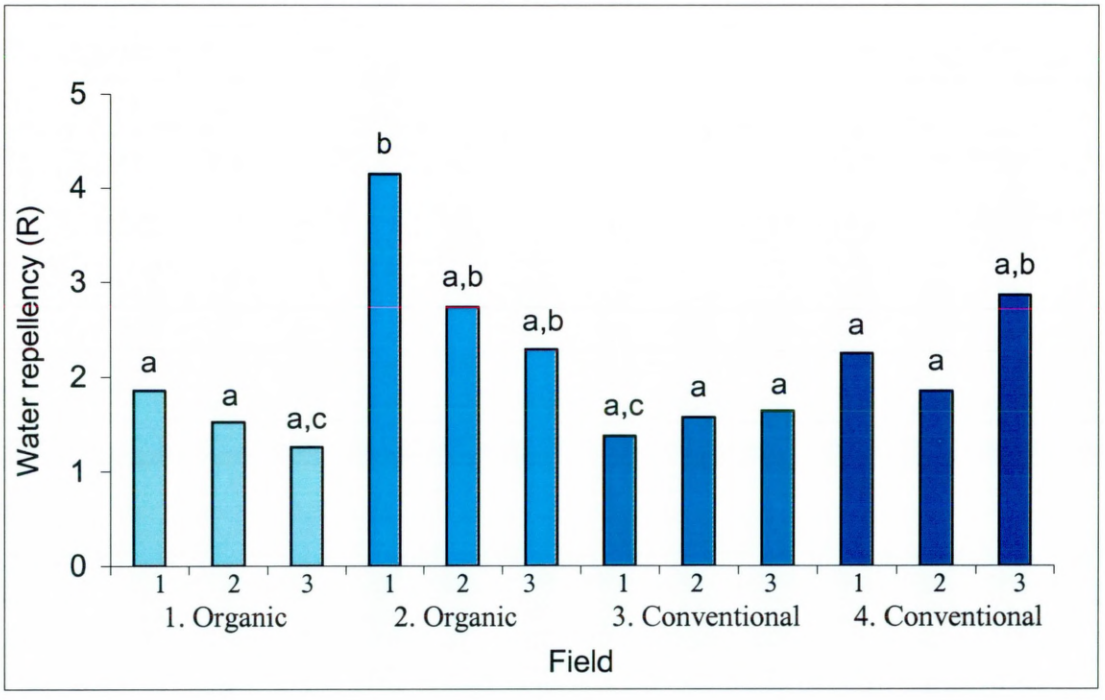


Fig. 5.9 Stockless arable organic soil, Terrington levels of water repellency, four fields were sampled with three different locations in the field that are represented as 1-3. Different letters denote significant differences (P< 0.01) between plots.

5.3.4 Correlations between measures for all field sites

Correlations between measures were assessed using a Pearson product moment correlation, the results of which are shown in Table 5.4.

Palace Leas: Significant positive correlations were detected between all measurements with the exception of the two repellency measurements, which were

not positively correlated (P>0.05). Reasonable correlations were detected between glomalin and ergosterol against water repellency. These significant positive relationships were stronger when comparisons were made with repellency measures of cores, rather than those measures made upon soil aggregates.

Broadbalk: Significant positive correlations were detected between all measures, with the exception of the correlation between glomalin and water repellency measured in aggregates (P>0.05). In contrast to this there was a positive relationship detected between glomalin (and ergosterol) and water repellency assessed upon soil cores (P<0.001). A relatively weak but significant, correlation was detected between the two water repellency measurements.

Terrington: No significant correlation between ergosterol and glomalin was detected (P>0.05). Significant positive correlations between glomalin and ergosterol against water repellency were detected. These correlations were however weaker than those detected under previous soils.

	Palace Leas			Broadbalk			Terrington	
	Ergosterol	Glomalin	Repellency (core)	Ergosterol	Glomalin	Repellency (core)	Ergosterol	Glomalin
Glomalin	0.398 0.018			0.596 <0.001			0.050 0.705	
Repellency (core)	0.523 0.01	0.643 <0.001		0.680 <0.001	0.647 <0.001		0.392 0.002	0.367 0.004
Repellency (aggregate)	0.421 0.013	0.498 0.003	0.223 0.204	0.497 0.001	0.223 0.166	0.369 0.019	N/A*	N/A*

Table 5.4 Correlations between measurements; top value is the Pearson correlation, whilst the number beneath is the associated P-Value. *No water aggregate water repellency measurements were completed for Terrington soils.

Plots of ergosterol and glomalin against water repellency for all data points are illustrated in Fig. 5.10a&b. Analysis of all data points resulted in the detection of significant correlations between glomalin, ergosterol and water repellency r= 0.69 and r=0.66, respectively (both P<0.001).

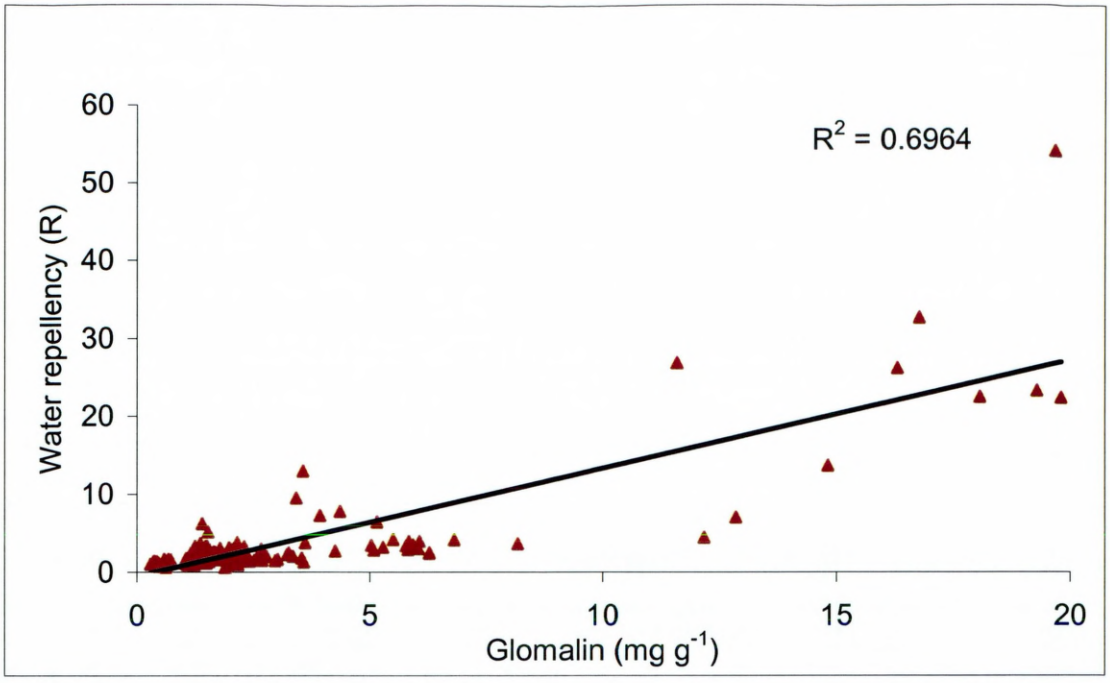


Fig 5.10a Correlation between glomalin and water repellency (measured on soil cores) all field sites included.

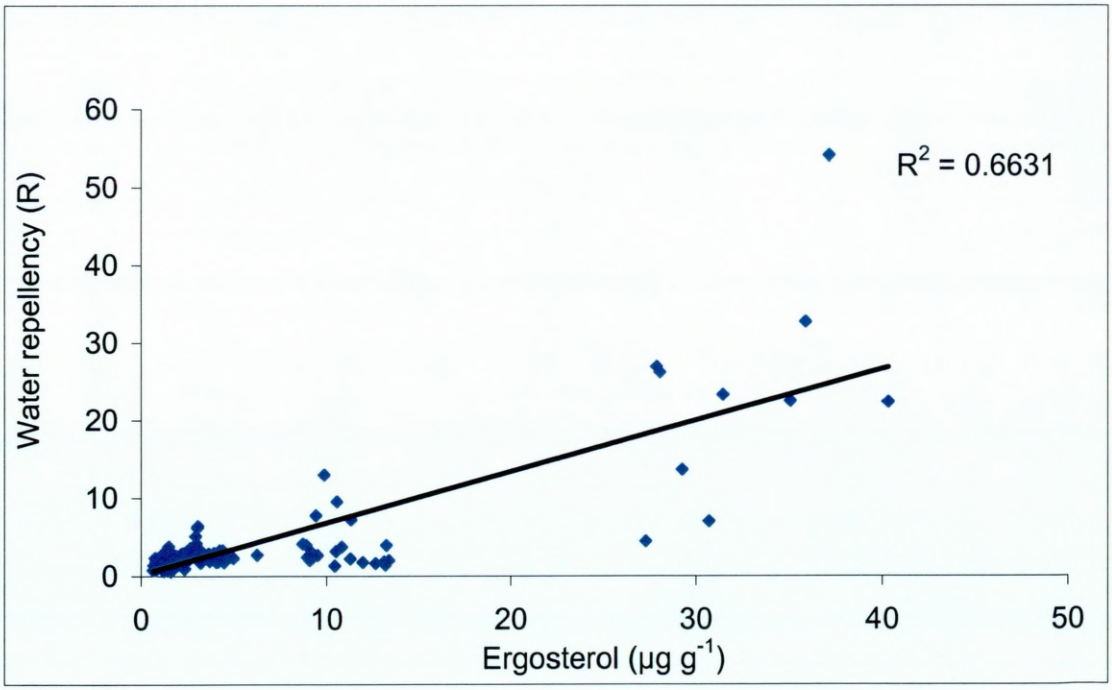


Fig 5.10b Correlation between ergosterol and water repellency (measured on soil cores) all field sites included.

5.4 Discussion

Nutritional additions to soils had a significant impact upon measures of fungal biomass (ergosterol), glomalin and water repellency.

Long-term grassland: Palace Leas

The Palace Leas field site allowed a unique opportunity to examine the long-term impact of nutritional treatments on fungi and water interactions upon soils that have remained unploughed since 1896. Given the history and background information on the site, and the undisturbed nature of the soil, there was the expectation that much of the fungal biomass would be mycorrhizal. Thus, nutritional inputs directly associated with mycorrhizal interactions would be likely to have the most significant impact upon the relationships observed. The effect of fertilizer treatments was most pronounced in this field site.

Although indicative of total fungal biomass, the increased concentrations of ergosterol (and glomalin) detected in plots 7 (N) and 11 (N&K) are likely to indicate increased AM fungal populations, which are more likely to thrive in P-limiting environments. Soils with high levels of soluble phosphate result in plants that are sparsely colonised or host no AM fungi (Russell, 1988). Data produced by the Palace Leas field site (Shiel, 2000b) show negligible levels of soluble P in plots 7 and 11. Joner (2000) demonstrated that plant growth was poor without mycorrhizal associations, most likely due to limited P uptake. However plants that received FYM grew well regardless of mycorrhizal associations. Hay yield was slightly lower in plots 7 & 11 and it is likely that plants in these plots are highly mycorrhizal dependent for their P intake. The application of 20 t ha^{-1} FYM will be a source of, on average 128 kg N, 17.6 kg P, 88 kg K, and will also act as a source of Ca, Mg, S and other micronutrients (Russell, 1988). The P and K applied from FYM will be as effective as non-organic fertilizers, whilst only a quarter of N from FYM is as effective as N supplied by non-organic fertilizers. Lower levels of ergosterol in plots amended with FYM may have been due to reduced mycorrhizal colonisation, as a result of increased nutritional inputs.

Changes in pH as a result of ammonium based fertilizers were also notable with plots 7 and 11 having the lowest pH compared to the other plots (Shiel, 2000b), with the application of FYM reported to either maintain or increase soil pH (Russell, 1988). No data was available quantifying percentage organic C or N.

High water repellency levels were detected in the Palace Leas field site in contrast to any of the agricultural soils (including those presented in Chapter 3). The high repellency levels were likely to be as a result of the undisturbed nature of this field site. With a possible build up of live (and dead) fungal hyphae and microbial exudates, indicated by both ergosterol and glomalin levels, which correlated with repellency measures.

The nutritional inputs on Palace Leas soils had considerable and significant influences upon the measurements made. The apparent reduction in fungal biomass related to P application is likely to be as a result of changes in mycorrhizal associations with plants. Ergosterol quantifies total fungal biomass and cannot distinguish between saprophytic and mycorrhizal species. Thus, the changes reported here require a further more comprehensive investigation of these soils to allows these factors to be fully explained. Further work is required to substantiate the proposed mechanisms behind the results presented, with previous researchers reporting low concentrations of ergosterol in AM fungi (Antibus and Sinsabaugh, 1993; Frey *et al.*, 1994). However, much of the work of both Antibus and Sinsabaugh (1993) and Frey *et al.* (1994) was completed on cultured organisms. With a large proportion of the fungal population remaining uncultured and un-characterised (Hawksworth and Rossman, 1997) the expression of ergosterol reported by Antibus and Sinsabaugh (1993) and Frey *et al.* (1994) may not hold true for some fungal species.

Long-term arable soil: Broadbalk field site

In a similar manner to the Palace Leas, Broadbalk field site allowed the opportunity to investigate long-term impacts of fertilizer regimes upon soil properties, with minimal changes to fertilizer and rotation treatments since 1843. The disturbance of the plots gave the expectation that, as with most conventionally managed arable soils

fungal biomass would be reduced (Beare *et al.*, 1992; Doran, 1980; Lupwayi *et al.*, 1998).

Broadbalk displayed significant effects of nutritional additions, but on a considerably reduced scale. Levels of glomalin, ergosterol and repellency were considerably lower compared to the grassland site. Glomalin values in wheat plots amended with FYM were statistically similar to the undisturbed grazed, and wooded plots. When comparing the data with the support information (Table 5.3), it is clear that the undisturbed plots share some similar characteristics to FYM amended plots, with similar levels of percentage organic C, % N and pH. The percentage organic C and N appeared to be good indicators of glomalin concentrations. In this case FYM clearly supplies equivalent to inorganic fertilizers, levels of N and organic matter (and K, P and Mg) to the soils, and has either a direct or indirect affect upon glomalin production or degradation. Rillig *et al.* (2001b) reported that glomalin could make up 4-5% of total soil C and N. In this highly disturbed system it is possible that inputting high levels of organic matter into the soil impedes, or slows, glomalin degradation. Six *et al.* (2000b) demonstrated that no-till systems had increased levels of organic matter in comparison to tilled systems. Broadbalk soils amended with FYM had equivalent levels of organic C compared to undisturbed soils, an unexpected property. It is possible that the application of FYM inputs greater levels of organic C than the wooded and grazed systems, even with the increased degradation of C as a result of tillage (Six *et al.*, 1999). Changes in the level of glomalin exudation due to fertilizer regime could have been as a result of different numbers of AM fungi. However, the significant impact of FYM upon glomalin levels is indicative of significant effects of a high carbon to nitrogen ratio (low N), which has been shown to stimulate polysaccharide production (Auer and Seviour, 1990). There may also have been differences in extra-cellular exudates produced by the other sections of the microbial community that were not measured. Roberson *et al.* (1995) also reported significant effects of N application upon microbial polysaccharide production, demonstrating that soil aggregation was associated with quantities of microbial extracellular polysaccharides rather than microbial biomass C (total microbial numbers). As AM fungi will be less prevalent in arable systems (McGonigle and Miller, 2000) the impact of bacterial activity (Beare *et al.*, 1992) and the associated polysaccharide production will be of

increasing importance in arable systems (Roberson *et al.*, 1991, 1995). This area merits further research.

The same marked changes in repellency observed in Palace Leas were not repeated in Broadbalk soils. Whilst significantly lower levels of repellency were observed under some of the wheat plots, differences between these and the undisturbed wilderness plots were not as marked as expected. The effect of continually producing the same crop without the addition of any nutritional amendments should amount to notable changes in soil measurements. Significant differences were reported between no fertilizer plots and nutritionally amended plots in fungal biomass, but there were no differences of this nature in measures of repellency. Like glomalin, differences in repellency were similar to the levels of organic C and N, giving an indication that other factors such as bacterial activity, or root influences may have a more considerable impact upon water repellency levels in this system. The non-significant differences in repellency may have been due to reduced soil structural stability, which has been reported around the roots of wheat, through increased levels of clay dispersion (Russell, 1988). Regardless of variations in measures of repellency, significant positive correlations between the measurements were still detected in this soil.

Increased concentrations of ergosterol detected in wooded and grazed plots were expected as these plots were not subjected to physical disturbance, which is known to reduce fungal biomass (Beare *et al.*, 1992; Doran, 1980; Lupwayi *et al.*, 1998; Young and Ritz, 2000). The concentrations of ergosterol in Broadbalk soils were much lower that those observed in Palace Leas; when comparing the two undisturbed plots (Broadbalk), ergosterol was detected at 4.2-4.4 $\mu g\ g^{-1}$ in wooded and grazed plots, whilst ergosterol was detected at 12.8 $\mu g\ g^{-1}$ in the equivalent Palace Leas plot (Plot 6, no nutrients added). Less supportive data was available for the Palace Leas field site and it is possible that there were considerable differences in organic C and N content between those plots or the differing vegetation type influenced the fungal populations. Some Broadbalk plots sampled had restricted pesticide application where, unlike other plots where no fungicide was applied. The effect of this upon measurements was not apparent; increased levels of fungal biomass would be expected in these plots, but this was not the case. There could have been non-target

effects of fungicide (Nannipieri *et al.*, 1994; Velvis, 1997); additionally the effects of the fungicide may have been short lived (McGovern, 2003 unpublished PhD thesis).

Stockless arable organic soil: Terrington

Treatments applied to the experimental field site Terrington have been established since 1990. In contrast to the previous field sites this could be considered a relatively new site. The nutritional additions to these soils did not cover as considerable a range as those of Broadbalk and Palace Leas soils. It could be could be hypothesised that differences would most likely to be detected between fields that received no nutritional input i.e. organic and those that received maximal nutritional impact i.e. conventional. The large and significant differences in measures detected in previous field sites were not observed in Terrington soils. Water repellency levels showed no distinct differences between fields. The same was true for glomalin with the exception of one conventionally managed field, which received the greatest nutritional inputs. Fungal biomass (ergosterol) was the only measure in this field site that differed significantly in concentration between fields. The highest concentration of ergosterol detected was in an organically managed plot, which received no nutritional input, but prior to sampling had cropped beans. No differences in ergosterol concentration were detected between the conventionally managed plot that received the greatest nutritional inputs, and an organically managed plot which received a phosphate amendment.

The lack of significant variation between plots in both glomalin and water repellency, and the low degree of correlation although significant and positive, indicates the positive correlations detected may have been the minimal relationship possible and in this case other factors not measured could potentially have more relevance upon soil water repellency.

Water repellency levels in cores and aggregates

Repellency levels differed when comparing measurements made upon intact soil aggregates and re-packed soil cores. Minimal error in the measurement should occur, as differences in the soil to infiltrometer tip contact will be overcome through sorptivity measures of both ethanol and water, with ethanol sorptivity unaffected by

contact (Letey, 1969). Intact aggregates were selected from the bulk soil; these aggregates must have been stable in the field, indicating that fungi and other extra-cellular polysaccharides may have been present on the outer side of aggregates or between aggregates (Kilbertus, 1980) in order to maintain soil stability, possibly through increased levels of repellency. In addition the migration of soluble repellent compounds during soil drying will result in increased surface hydrophobicity (Czarnes *et al.*, 2000). Soil cores also consisted of homogenised soil, whilst aggregates were randomly selected from field soils and therefore subject to an increased degree of spatial variation. The process of sieving soil may abrade particle surfaces thus removing some hydrophobic coatings.

Correlations between repellency and other measures appeared to be stronger using re-packed cores. However, if glomalin is not readily broken down or mobile in soil as proposed by Rillig *et al.* (2001b), differences in measures of water repellency should have little bearing upon the relationship between the two measures.

Overview of the impact of nutritional additions at the field scale

The literature investigating the effects of land management upon soil fungal biomass and soil water relations is wide ranging, but limited. Few studies investigate the impact of land management on the combination of microbial, chemical and structural changes in soil. High N fertilization can have a negative effect upon extracellular polysaccharide (EPS) production, additionally causing a reduction in saturated hydraulic conductivity and aggregate slaking resistance (Roberson *et al.*, 1995). Roberson *et al.* (1995) postulated that high concentrations of N fertilizer applied to soil might saturate the system affecting microbial processes and populations. Roberson *et al.* (1995) proposed that the effects of tillage would reduce the role of fungi in the aggregation process, further proposing that the importance of fungi was greater in reduced or no-tillage systems, a factor previously reported by Beare *et al.* (1992). Donnison *et al.* (2000) reported that ergosterol content was higher when organic fertilizers were applied (manure), rather than inorganic. A mechanism apparent in Broadbalk soils (under the restricted pesticide regime), but was not applicable to Palace Leas soils, where minimising P application had a more significant influence over fungal biomass than any other factor. It can therefore be presumed that variations in fertilizer applications will affect soils differently

depending on the level of disturbance applied (among other factors). The resulting impact upon fungal populations will have direct and indirect consequences for soil water relations.

5.5 Conclusions

The assessment of soils subjected to various long-term land management practices resulted in the detection of considerable direct and/or indirect affects upon fungal biomass, glomalin concentration and water repellency levels.

High levels of ergosterol detected in the grassland field site indicate that fungi were most likely to be the dominant microorganism, and have a significant impact upon soil water repellency. However, in the highly disturbed arable plot of Broadbalk, good correlations between ergosterol, glomalin and water infiltration were still detected. This was indicative that the correlations may always be present within a soil, but the extent to which these relationships impact upon a soil's stability will be interlinked with other factors, such as nutritional additions, organic C, disturbance and vegetation type. There may be evidence of a threshold effect of both glomalin and ergosterol upon water repellency, where below a set level no positive relationship is detected whilst above a certain level there is no additive effect of either fungal biomass or glomalin to this relationship. An investigation of the concentration of ergosterol expressed in AM fungi would give greater insight into these results, as would a further biochemical analysis of microbial populations.

Further work establishing how differing fertilizer regimes change not only total fungal biomass but also fungal population dynamics, and additionally how EPS production is affected, allowing a greater understanding of how chemical and biological processes interact with soil stability mechanisms.

Chapter 6: General discussion and conclusions

The aim of this thesis was to investigate the impact of fungi on the structure-water relations in soil. Using a range of biophysical diagnostic tools, I examined the role of total fungal biomass and an AM fungal exudate on the hydrophobicity of soil and the resultant stability of the soil. For the first time, I examined the impact of biological activity on the fine (micrometer) scale soil structure. Finally, utilising two unique sites, representing the oldest experimental grassland and conventional sites in the UK, Palace Leas and Broadbalk, the laboratory-based observations were tested at the field scale.

Since the discovery of glomalin in 1996, over 30 papers have been published on this protein ranging from the production, effects of tillage and plant species, to the carbon content of this protein (ISI Web of Science). Much of the research has, however, concentrated on reporting on the relationship between glomalin and soil stability, with many of the publications from the USA, where significant effort is been spent on attempting to link the exudation of glomalin to soil structural stability and carbon inputs into the soil ecosystem.

As discussed in this thesis, positive correlations between total glomalin concentration and soil aggregate stability have been reported (Wright and Anderson, 2000; Wright *et al.*, 1999), with researchers describing the properties of glomalin to be glue-like and hydrophobic (Wright and Upadhyaya, 1998). The use of aggregate stability measures assumes that these properties either directly or indirectly affect soil structural stability, and therefore increases in aggregate stability are directly accredited to equivalent changes in glomalin concentration. The only mechanism offered to explain these results and hypotheses has been that "*glomalin contributes to hydrophobicity of soil particles*" and may affect water drainage (Wright and Upadhyaya, 1998). Prior to the work in this thesis no assessment of this fundamental mechanism had been investigated.

In Chapter 2 the application of a spatial investigation was used to assess for a relationship between glomalin and soil water repellency. Without the development of significant differences in glomalin concentration between the two treatments, no correlation between water repellency and glomalin concentration was detected.

There were however, significant increases in water repellency, which may have been induced through microbial or root activity (Chan, 1992; Jaramillo *et al.*, 2000; McGhie and Posner, 1980; Moore and Blackwell, 1998; White *et al.*, 2000). However, the resulting differences in repellency could not solely be attributed to either root effects or microbial activity without the application of further measurements. Therefore an experimental set-up, with a comprehensive root system, was selected and prepared in a way that root effects could be separated from bulk soil (Chapter 3). Progressing from Chapter 2 an additional measure of fungal biomass was applied to assess how this may contribute to water relations. In addition to measures of water repellency, an assessment of aggregate stability was applied, as some researchers report impeded soil wetting with increased aggregate stability, with Caron *et al.* (1996) and Guggenberger *et al.* (1999), proposing increased hydrophobicity as a mechanism for aggregate stabilisation.

Prior to experimental perturbations, all structures greater than 2 mm were destroyed by sieving. Therefore, reported changes in structures greater than 2 mm were a direct result of the formation of new aggregates. The active re-aggregation through biological or physical processes of soils was indicated by the general decline in structures <2000 μm. Increases in macroaggregates >2000 μm were reported in all of the treatments, but were most pronounced in the rooted region (planted inner). Miller and Jastrow (1990) and Thomas *et al.* (1986) both reported correlations between root mass/length and proportion of macroaggregates. Although no measure of root length was made in the experimental set-up presented in Chapter 3, the most significant increases in aggregates >2000 μm (in both soils) throughout the incubatory period was in the rooted zone, were root mass/length undoubtedly increased over time. It would seem that roots provide the mechanical framework in the initial stages of aggregation, physically entangling soil particles, with further indirect effects from roots adding to soil aggregation processes (Jastrow *et al.*, 1998, Watt *et al.*, 1993).

One of the indirect affects of plant roots is increased microbial activity as a result of associated organic C inputs (Golchin *et al.*, 1994; Jastrow *et al.*, 1996). However, the impact of organic C from root material could be limited with Tisdall and Oades

(1982) proposing that less readily available organic C from material such as roots (and cellulose based substrates) allows for gradual increases in aggregates (over a period of months). More readily available C sources from plants such as extracellular polysaccharides have a more immediate affect, inducing changes in aggregate stability within a matter of weeks (Tisdall and Oades, 1982). Wetting and drying processes associated with plant roots as a result of evapotranspiration can also have positive effects upon soil aggregate stabilisation (Denef *et al.*, 2001; Denef *et al.*, 2002; Utomo and Dexter, 1982).

Minimal differences in fungal biomass between planted inner (rooted), outer planted (no roots but within root zone) and unplanted treatments were detected. Therefore the increased proportion of aggregates >2000 μm detected under planted (inner) conditions were likely to be a direct root effect, resulting in the physical entanglement of soil particles. In addition, indirect effects such as root exudates or localised wetting and drying would affect soil in the direct vicinity of roots (planted inner) and in the bulk soil (planted outer). However, the increases in aggregates >2000 μm present in non-rooted regions of the soil could not be attributed to root activity and resulted as a direct consequence of increases in fungal biomass, with fungal hyphae aggregating soil (Drury *et al.*, 1991; Haynes and Francis, 1993; Monlope *et al.*, 1987).

Similarly to increases in macroaggregates (>2000 μm), significant increases in water repellency were reported, for all treatments in both soils, with increases that could be directly correlated to increases in fungal biomass. The hypothesised process proposed by both Caron *et al.* (1996) and Guggenberger *et al.* (1999), that if wetting rate is impeded then the stability of a soil aggregate is increased, was confirmed. Increased repellency in Bullion Field soil under the planted treatment (which was greater than Lab Field soil), was matched also by a greater proportion of aggregates >2000 μm detected in Bullion Field soil. As there were minimal differences in fungal biomass between Lab Field and Bullion Field soil the differences in water repellency and aggregates >2000 μm must be as a result of another factor, with increased glomalin concentrations also detected in Bullion Field soil. The difference between the soils was probably due to the differences in glomalin, or another unmeasured factor such as other microbial exudates.

At first glance, the results of Chapter 2 and 3 are inconsistent. Whilst no relationship between glomalin and water repellency was reported in Chapter 2, a significant positive relationship was subsequently detected in Chapter 3 in Bullion Field soil (but not Lab Field soil). Lower levels of glomalin were detected in the Lab Field soil in comparison to the Bullion Field soil. Under these conditions it is proposed that this soil had inherently lower background levels. Thus, any build-up of glomalin in the Lab Field soil throughout the incubatory period may not have been at a significant enough concentration to have a positive influence upon soil water repellency.

The detection of a positive correlation in Bullion Field soil in Chapter 3, but not Chapter 2, may be due to differences in the experimental set-up. Chapter 2, had no AM fungal inoculum seeded in the soil cores, and the development of an AM fungal population was reliant on the establishment of the *in situ* soil inoculum. The results of root staining positively identified the presence (but not species) of AM fungi after 34 days incubation. The experimental set-up in Chapter 3 differed with experimental cores being inoculated with AM fungal spores. This may have induced differences between the two experimental set-ups thus accounting for the discrepancy in correlation. The un-inoculated soil would develop a mixed population of AM fungi, whilst the soil inoculated with *Glomus mosseae* would be likely to have this fungus as the dominant species. Wright and Upadhyaya (1996) reported that different fungal species might produce different concentrations of glomalin. Additionally, the inoculation of the soil would allow for a more rapid establishment of an AM fungal population. These factors may contribute to the differences in correlations between the two systems. In Chapter 2 there is the possibility that the spatial variation in water repellency levels identified in the planted samples could mask a correlation. The different plant types alone may have induced variations in repellency, again masking a correlation between glomalin and water repellency, with a different plant type, and therefore root system in each of the studies. Finally, the increased sample numbers in Chapter 3 would undeniably increase the possibility of the detection of even a weak relationship.

Changes in soil stability and fungal biomass as a result of incubation presented in Chapter 3 illustrate how the stability of soil changes at the millimetre scale. To date it was unknown if these changes relate to micro- and meso-scale pore space differences. Chapter 4 involved the novel experimental analysis of soil aggregates through X-ray computed tomography. In my knowledge, this is the first time X-rays of such energy have been used to analyse soil structural differences to a resolution of ≈4 μm. The analysis resulted in the detection of differences in percentage porosity and the spatial distribution of pores between the various treatments. Large and significant changes were detected in soil aggregate structural make-up after a short incubatory period of 30 days.

The lack of differences in both porosity and the spatial distribution of pores between day zero samples and unplanted (30 day incubation) samples led to the proposal that changes were induced as a result of both direct and indirect plant activity, with the most likely effect through wetting and drying. The influence of wetting and drying upon soil has been investigated by Czarnes *et al.* (2000), Denef *et al.* (2001) and Denef *et al.* (2002), with Denef *et al.* (2001, 2002) reporting increased stability as a result of exposure to wet~dry cycles. Kemper *et al.* (1987) reported that drying decrease the distances between particles, causing increases in associations between particles, and causing more stable associations to form between particles.

The increased heterogeneity of pore spatial variation in planted samples matched factors proposed by Guidi *et al.* (1985). They stated that the wetting and drying of an initially homogenous soil (in this case sieved) could result in the "heterogenisation" of pore distribution. The re-arrangement of soil particles in a relatively short period of time demonstrates the transient nature of soil structures when given the potential to change. Without further investigation the exact mechanisms behind these changes in structure will remain unsubstantiated.

More specific investigations are required to study abiotic physical interactions in soil structure. The potential of the X-ray computed tomography methodology used is such that differences in soil structure as a result of various treatments may be visualised without the interference of disturbance. The method can also be applied at

varying scales, appropriate for the associated process of interest. Clearly the physical interactions involved in soil structural genesis cannot be overlooked. The combination of the rearrangement of soil aggregation at the micro-scale, and biological impacts upon sorptivity, only serve to emphasise the importance of the biophysical and chemical interactions within a functioning soil ecosystem.

Comparing the results of the porosity examination with the equivalent repellency levels reported in Chapter 3 highlighted a possible discrepancy between porosity and water repellency. Increases in pore space should hypothetically provide an increase in void space in the soil for water flow. This was not the case, with no differences in water repellency reported between different extremes of micro-porosity. This indicated that other factors might have had a significant impact upon water relations. The release of extra-cellular polysaccharides (EPS) into the soil results in the formation of sheaths of EPS that form associations with clay minerals. Chenu (1993) reported that these associations could exhibit properties that may affect soil functions "*buffering against water potential fluctuations*". It is possible in the case of the results presented, the volume of pore space available for the infiltration of water was not the defining factor for water repellency levels. Levels of EPS, or other hydrophobic substances, not measured may have controlled low levels of water repellency. Visualisation of these sources of hydrophobicity through scanning electron microscopy (SEM) and transmission electron microscopy (TEM), as reported by Chenu (1993) paired with small-scale measures of infiltration may allow further understanding of this.

The effect of changing fungal biomass upon soil water processes was further examined in Chapter 5, where the impact of disruption and fertilizer application upon soil water repellency and fungal biomass was investigated at the field scale. The use of well-established field sites gave a unique opportunity to investigate long-term nutritional impacts upon soil processes, developing on the work carried out in the laboratory. The most significant impact of fertilizer treatments was detected in grassland soils, where large concentrations of both ergosterol and glomalin were isolated, alongside considerable levels of water repellency. The fungal indicators measured indisputably had a direct impact upon water repellency, but in a grassland system increased levels of organic C would be expected in contrast to arable soils, as

the dense root mass in the soil will continually grow and breakdown. The presence of increased organic matter can also induce increased soil hydrophobicity (Chenu *et al.*, 2000).

Hallett and Young (1999) detected changes in water repellency as a result of nutritional additions with a corresponding change in microbial activity. In the case of the results presented here, the high levels of repellency are likely to be as a consequence of the presence of considerable fungal biomass (and associated exudates). The relationship between fungal biomass-glomalin-water repellency continued in the agricultural soils even with reduced levels of both glomalin and fungal biomass. The correlations between fungal biomass and glomalin against water repellency did not become stronger under any conditions.

The influence of different fertilizer regimes appeared to have a varying influence upon the measurements made dependent on the land management system. Soil disturbance will change soil microbial populations (Doran, 1980; Lupwayi *et al.*, 1998; Young and Ritz, 2000), thus nutritional amendments will affect microorganisms differently dependent on the land management system.

The correlations reported between glomalin and fungal biomass against water repellency appeared to reach an optimum above which the correlation fails improve. The influence of possibly both glomalin and fungal biomass may only have an effect at a certain concentration, a possible threshold effect below which there is no or little influence of glomalin (as indicated by the differences in correlations reported in Chapters 2 & 3), and above a certain concentration there is no increasing influence (illustrated in Fig. 6.1). A similar threshold effect was found by Read *et al.* (2003).

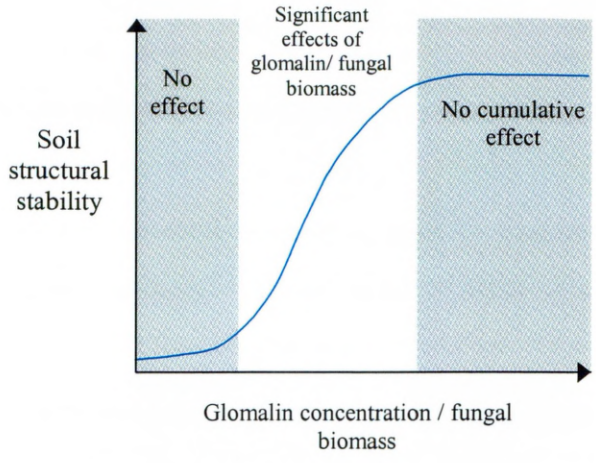


Fig. 6.1 Schematic of proposed relationship between glomalin concentration and soil stability, which can be, assessed either as aggregate stability of soil water repellency.

This factor paired with the application of various inappropriate measures of soil stability, may explain why some researchers have reported mixed effects of glomalin upon soil stability (Borie *et al.*, 2000; Franzluebbers *et al.*, 2000). Additionally much of the research may ignore the impact of numerous factors each having a level of covariance. Whilst glomalin and fungal biomass may have an effect upon soil structure these indicators are unlikely to be the sole factors responsible for changes in soil structural stability. These factors will instead contribute to soil stability alongside roots, fungal hyphae, other soil microbial activity, and various soil physical processes such soil moisture induced re-organisation of soil particles (Semmel *et al.*, 1990).

The investigation of soil structure is necessary, as soil is a valuable resource that is essential to environmental buffering and the sustainable production of crops. Assessment of the direct and indirect affects associated with agricultural practices are important to detect changes in soil that may be detrimental, in either the short or long-term. Investigations of soil physical stability using traditional measurements based on aggregate stability assessment can now be paired with increasingly advanced techniques that allow more applied measurements that are directly associated with processes in the field. Utilising technology that allows the visualisation of physical changes in soil without considerable disturbance will add

increasing value to soil assessments. Changes in pore structure observed in lab scale investigation may occur in the field. Understanding of the processes associated with these changes may present the potential to "engineer" more favourable structural environments for specific microorganisms and plants, ultimately allowing a greater understanding of the numerous complex interactions that influence soil structure.

6.1 Future research

As a result of the research presented in this thesis, there are areas in this field that I feel require further investigation:

- Further investigation of the utility of ergosterol as measure of total fungal biomass, present in all fungal hyphal membranes, but proposed to be present in considerably smaller quantities in AM fungi (Antibus and Sinsabaugh, 1993; Frey *et al.*, 1994). When used in grassland soils much of the fungal population will be mycorrhizal, therefore establishing the concentration of ergosterol expressed in these fungi will further enhance the usefulness of this fungal biomarker.

- The positive relationship between fungal biomass and water repellency and aggregation (of aggregates >2000 μm) was strong throughout the experimental set-up, which ran for only 30 days. No substrate was added to the system, therefore increases in fungal biomass would not continue indefinitely without additional fresh input of substrate. After increased incubation, how would these correlations continue, would there be legacy effect of fungal hyphae? Additionally in a longer-term experimental set-up the effects of root age may be assessed, with root material at some point becoming a substrate source, which could allow increased microbial activity and in turn increased soil stability.

- The proposed threshold effect of both fungal biomass and the AM fungal exudate glomalin requires more controlled experimental analysis in order to

evaluate a causative impact upon water repellency of both fungal biomass and glomalin, as opposed to correlations. Investigations upon soils not exposed to AM fungi or experiments in sand would allow the establishment of minimal levels of fungal biomass and concentrations of glomalin. This would allow the assessment of structure and water flow at various levels of fungal colonisation and or glomalin concentration establishing at what level there is a significant effect without the interference of other factors. The possibility of adding various concentrations of glomalin extracts to sand in order to assess the direct impact upon repellency should be considered. Additionally, measuring the contact angle of glomalin extracts at differing concentrations would give valuable information on the wettability of this extract in relation to protein content.

- The results of the 3D X-ray analysis merit a more comprehensive investigation, using bigger samples to allow further analysis of solid matter spatial differences. Changing the scale of investigation may also allow different changes in structure to be associated with different factors. Abiotic factors may dominate at the micron scale (e.g. resulting in the re-orientation of clays at ≤ 2 μm) whilst biotic factors may influence at an increased scale by changing pore structure and connectivity. Sampling the soil after a shorter incubation period will give further indication of when specific changes occur. The proposed influence of wet~dry cycles upon soil porosity requires investigation under the application of controlled wet~dry cycles upon unplanted soil samples. Further to this, the completion of the experiment upon a tension table would enable the experiment to be maintained under constant moisture content.

- The application of more comprehensive geostatistics to the 3D X-ray data sets would establish if pore connectivity and tortuosity differs between treatments, and how these structural changes affect soil properties.

- Subcritical water repellency levels are likely to be related to levels of hydrophobic substances and pore space. The work presented in this thesis

did not detect a relationship between porosity and repellency. A combined assessment of these factors utilising X-ray analysis for visualisation of physical structure and SEM or TEM to visualise hydrophobic soil coatings could be paired with infiltration measurements to attempt to visualise specific areas of soil hydrophobicity. Combining the visualisations before, during and after water infiltration measurements will allow a greater understanding of how water flow and soil structure are controlled under a variety of biological perturbations.

- Characterisation of hydrophobic compounds by investigating their chemical composition would allow a greater understanding of why these substances are released by microorganisms (and plants). Additionally, this methodology would lend itself to understanding the rate of degradation of particular compounds and how they affect soil hydrophobicity.

- A more comprehensive investigation of how fertilizer additions to soils affect microbial populations would allow a greater understanding of how arable practices affect soil structure, assessing how fertilizer additions may affect soil microbial exudation and which fungal species are most susceptible to changes. Additionally, which fungal species in the field are predominant, and more specifically which are responsible for soil aggregation and water repellency changes. Further investigation of how fertilizers react in different systems investigating the difference between disturbed (tilled) and undisturbed (reduced tillage) systems.

- The correlations reported throughout the thesis were assessed independently of one another. Jastrow *et al.* (1998) reported many of the factors associated with soil structure have complex interactions and covariance between them. Therefore, further assessments of this nature should apply more advanced analysis, possibly weighting factors on their importance in particular systems. This would give added value to data allowing a greater understanding of inter-relationships in different systems.

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